VOL 233, NO. 5

The Journal OF Biological Chemistry

NOVEMBER 1958

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All manuscripts should be addressed to Dr. John T. Edsall, *Editor, The Journal of Biological Chemistry*, 10 Divinity Avenue, Harvard University, Cambridge 38, Massachusetts.

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A list of approved abbreviations, which may be used without further definition in articles in the *Journal*, will be found in the article on "Use of Abbreviations" on pages 3 and 4. Other abbreviations should be used sparingly, and must be defined by each author in a footnote at the point where the first such abbreviation occurs. Ordinarily no abbreviations should be used in summaries. For further details, see "Use of Abbreviations."

Separate sheets should be used for the following: (*a*) title, (*b*) author(s) and complete name of institution or laboratory, (*c*) running title, (*d*) references, (*e*) footnotes, (*f*) legends for figures, (*g*) tables, and (*h*) other subsidiary matter. When an elaborate mathematical or chemical formula (one which cannot be printed in single horizontal lines of type) appears in the text, a duplicate of it should be typed on a separate sheet. All such supplementary sheets, except the title, author(s), and running title pages, should follow the text, and all sheets should be numbered in succession, the title page being page one. Manuscripts that do not conform to these instructions will have to be cut and rearranged by the printer so that the matter to be set in different type sizes can be separated.

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A desirable plan for the organization of a paper is the following: (*a*) introductory statement, with no heading, (*b*) "*Experimental Procedure*" (or "*Methods*"), (*c*) "*Results*," (*d*) "*Discussion*," (*e*) "*Summary*," (*f*) "*References*." The approximate location of the tables and figures in the text should be indicated in the margin. Any general acknowledgments that are to be made should be placed after the *Summary*, just preceding the *References*. Mention of more specific instances of acknowledgment may be made in footnotes.

1. The introductory paragraphs should state the purpose of the investigation and its relation to other work in the same field, but extensive reviews of the literature should not be given. A brief statement of the principal findings is helpful to the reader.

2. The description of the experimental procedures should be as brief as is compatible with the possibility of repetition of the work. Published procedures, unless extensively modified, should be referred to only by citation in the list of references.

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6. The references should conform to the style used in current issues of the *Journal*. In the case of books, the authors' names with initials, the title in full, the edition if other than the first, the publisher, the place of publication, the year of publication, and the page should be cited, in this order. Responsibility for the accuracy of bibliographic references rests entirely with the author; all references should be confirmed by comparison of the *final manuscript* with the original publications. Mention of "unpublished experiments," "personal communications," etc., must be made in footnotes, and not included in the *References*. References to papers which have been *accepted* for publication, but which have not appeared, should be cited just as other references, with the abbreviated name of the journal followed by the words "in press." It is advisable that copies of such papers be submitted to the Editors whenever the findings described in them have a direct bearing on the paper for publication.

7. If the paper submitted is one of a series, the immediately preceding paper of the series should be included in the references, and identified as such, either in the text or in a footnote near the beginning of the paper.

Chemical and Mathematical Formulas

Reference in the text to *simple* chemical compounds may be made by the use of formulas when these can be printed in single horizontal lines of type. The use of structural formulas in running text should be avoided. Chemical equations, structural formulas, and mathematical formulas should be centered between successive lines of text. Unusually complicated structural formulas or mathematical equations which cannot conveniently be set in type should be drawn in India ink on a separate sheet in form suitable for reproduction by photoengraving (examples, *J. Biol. Chem.*, **228**, 612, 630, 714, 753 (1957)).

Tables

For aid in designing tables in an acceptable style, reference should be made to current issues of the *Journal*. A table should be constructed so as to be intelligible without reference to the text. Only essential data should be tabulated. Every table

should be provided with an explanatory caption, and each column should carry an appropriate heading. Units of measure must always be clearly indicated. If an experimental condition, such as the number of animals, dosage, concentration of a compound, etc., is the same for all of the tabulated experiments, this information should be given in a statement accompanying the table, and not in a column of identical figures in the table.

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Only in exceptional cases, the necessity for which must be clearly demonstrated, may the same data be published in two forms, such as in a table and a line figure.

Illustrations

The preparation of illustrations is particularly important, and authors are requested to follow carefully the directions given below. In case of doubt, the Editorial Office will gladly supply specific information.

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Use of Abbreviations

The *Journal* recognizes the utility and convenience of some nonstandard abbreviations for chemical substances, particularly in equations, tables, or figures requiring several unwieldy terms in close or repeated context. The *Journal* therefore will accept a limited use of abbreviations of specific meaning as defined below. However, in order that scientific writing be comprehensible to the reader, clarity and unambiguity of expression must remain prime considerations.

Titles and summaries, which are most often translated into foreign languages, should be free of abbreviations, and abbreviations should be minimized in the introductory sections. In the remainder of the manuscript, abbreviations may be used in the text sparingly and only if advantage to the reader results. Chemical equations, which traditionally depend upon abbreviations, may utilize an abbreviation for a term which appears in full in the neighboring text. This latter procedure is also permissible in summaries, when necessary to avoid excessively cumbrous expressions, as in equations or polymer structures. Such essential abbreviations in summaries would be defined therein when first used; *e.g.* flavin adenine dinucleotide (FAD).

Formulation of Abbreviations—Abbreviations other than those listed or defined below should be in accordance with the following principles:

The number of these introduced per article should be limited (3 or 4); none should be introduced except where repeated use is required (see above). Three letters are considered optimum in length. The formation of words by these letters or duplication of an accepted abbreviation is to be avoided. Where a number of derivatives, salts, or addition compounds may be formed, the root or common basic structure should be the one abbreviated so that appendages may be hyphenated to it (*e.g.* AMP-sulfate, acyl-AMP). It is suggested that abbreviated names be hyphenated throughout, even where the full name is not (*e.g.* glucose-6-P). Structural analogues of a given compound are not to be abbreviated as if they were derivatives of that compound (*e.g.* the nicotinic acid analogue of DPN). Accepted symbols for elements or radicals are recommended where applicable (*e.g.* glycero-P for glycero-phosphate, P-glycerate for phosphoglycerate).

Names Ordinarily Not To Be Abbreviated—Names of enzymes (except when named in terms of an abbreviation, *e.g.* glucose-6-P dehydrogenase, ATPase, but glucose-6-phosphatase; RNase, DNase), pyridoxal, pyridoxamine, deoxypyridoxine, thiamine, cocarboxylase, pantothenate, folic acid, pteroylglutamate, trichloroacetic acid, perchloric acid, the tricarboxylic acid cycle and members thereof. Abbreviations for pteroyl-L-glutamic acid derivatives (but not the substance itself) may use PGA for the parent compound (*e.g.* PGA-H_4; N^5-formyl PGA-H_4; hydroxymethyl (or CH_2OH-) PGA-H_4). (Note that pteroyl-L-glutamic acid is one member of the class of compounds known collectively as the folic acids.) However, since PGA has also been used by some authors to denote phosphoglyceric acid or polyglutamic acid, it is essential that this abbreviation be defined in a footnote in each paper in which it is used.

Accepted Abbreviations—The abbreviations in the list given below, may be used without definition. Other abbreviations should be defined in a single footnote at the point of introduction of the first one. Accepted abbreviations are as follows (*cf. Biochem. J.*, **66**, 8 (1957)):

DPN (or DPN^+), DPNH	diphosphopyridine nucleotide and its reduced form
TPN, TPNH	triphosphopyridine nucleotide and its reduced form
FAD, $FADH_2$	flavin adenine dinucleotide and its reduced form
NMN	nicotinamide mononucleotide
GSH, GSSG	glutathione and its oxidized form
CoA, acyl-CoA	coenzyme A and its acyl derivatives (*e.g.* acetyl, etc.)
AMP, GMP, IMP, UMP, CMP	the 5′-phosphates of ribosyl adenine, guanine, hypoxanthine, uracil, cytosine
2′-AMP 3′-AMP (5′-AMP), etc.	the 2′-, 3′-, (and 5′-, where needed for contrast) phosphates of the nucleosides
ADP, etc.	the 5′(pyro)-diphosphates of adenosine, etc.
ATP, etc.	the 5′(pyro)-triphosphates of adenosine, etc.
deoxy-AMP(dAMP, dGMP, dIMP, dUMP, dCMP, dTMP)*	the 5′-phosphates of 2′-deoxyribosyl) adenine, etc.

Phosphorylated derivatives may be depicted as phosphate derivatives of the parent compounds with P- or -P representing phosphate, as in glucose-6-P, P-glycerate, glycerol-P, P-enolpyruvate, PP-ribose-P (ribosylpyrophosphate 5-phosphate).

RNA, DNA	ribonucleic acid, deoxyribonucleic acid
RNase, DNase	ribonuclease, deoxyribonuclease
UDP-glucose, UDP-galactose, etc.	uridine diphosphate glucose, galactose, etc.

For Diagrammatic Representation of Polymers or Sequences—(*a*) For the amino acid residues in polypeptides, the residue with the free alpha-amino group (if one is present) to be understood to be at the left of the sequence as written (*Ann. Rev. Biochem.*, **16**, 224 (1947)):

Gly, Ala, Val, Leu, Ileu	glycyl, alanyl, valyl, leucyl, isoleucyl,
Pro, Phe, CySH, CyS-Met,	prolyl, phenylalanyl, cysteinyl, half-cystyl, methionyl,
Try, Arg, His, Lys, Asp, Glu,	tryptophyl arginyl, histidyl, lysyl, aspartyl, glutamyl,
Glu-NH_2, Asp-NH_2, Ser,	glutaminyl, asparaginyl, seryl,
Thr, Tyr, Hypro, Hylys	threonyl, tyrosyl, hydroxyprolyl, hydroxylysyl

These symbols should be separated from each other by periods (*e.g.* Gly.Val.Asp.Ser). Groups of residues of unknown se-

* When it is necessary to use d for deoxy, it should be so defined. The full prefix requires no such definition.

quence may be enclosed in parentheses and separated by commas. These abbreviations should be used only for amino acid residues in peptide linkage; never for the free amino acids, the names of which should be written out in full.

(*b*) For the polysaccharides (*J. Chem. Soc.*, **1952**, 5121; *Chem. Eng. News*, **31**, 1776 (1953)):

G,† Fru, Rib, deoxy-Rib (dRib), etc.*	glucose, fructose, galactose, ribose, deoxyribose, etc.

(These abbreviations are to be used only for derivatives or polymers of the monosaccharides, not for the free monosaccharides themselves, the names of which should be written out in full.) When it is necessary to indicate furanose, pyranose, etc., the letter *f* or *p*, following the saccharide abbreviation, may be used. Thus, Rib*f* for ribofuranose. To indicate a uronic acid, the suffix A, for acid, may be used. Thus, GA for glucuronic acid, GalA for galacturonic acid. To indicate a 2-amino-2-deoxysaccharide, the suffix N is added to the parent saccharide symbol, and an *N*-acetyl derivative is shown by NAc. Thus, GalN for galactosamine, GalNAc for N-acetyl galactosamine. Configuration symbols (L-, D-) may be used as prefixes. Each residue is separated from the next by a hyphen; thus, UDP-GalNAc.

† Confusion between G for guanosine and for glucose, if it occurs, should be avoided by the use of one name in full.

(*c*) For polynucleotides of specific structure, the letter p to the left of the nucleoside initial indicating a 5′-phosphate; the letter p to the right, a 3′-phosphate:‡ Thus, for polyribonucleotides (A, G, etc., representing the nucleosides of adenine, guanine,† etc.):

pApG	5′-*O*-phosphoryl-adenylyl-(3′-5′)-guanosine or guanylyl-(5′-3′)-adenosine 5′-phosphate
ApGp	adenylyl-(3′-5′)-guanosine 3′-phosphate
ApG-cyclic-p	adenylyl-(3′-5′)-guanosine 2′:3′-phosphate

for polydeoxyribonucleotides:*

d-pApGpT	5′-*O*-phosphoryl-deoxyadenylyl-(3′-5′)-deoxyguanylyl-(3′-5′)-deoxythymidine, or deoxythymidylyl-(5′-3′)-deoxyguanylyl-(5′-3′)-deoxy-adenosylyl 5′-phosphate.

‡ For further examples of this system of abbreviation see, for instance, Heppel, Ortiz, and Ochoa, *J. Biol. Chem.*, **229**, 679, 695 (1957).

Units of Measurement

Units of Mass

kilogram	kg.
gram	gm.
milligram	mg.
microgram	μg. (*not* γ)
millimole	mmole (*not* mM)
micromole	μmole (*not* μM)

Units of Concentration

molar (mole/liter)	M
millimolar	mM
micromolar	μM

Units of Length, Area, Volume, etc.

meter	m.
centimeter	cm.
millimicron	mμ
Angstrom (10^{-8}cm.)	A
square centimeter	$cm.^2$
cubic centimeter	cc., or $cm.^3$
liter	l.
milliliter	ml.
microliter	μl. (*not* λ)
sedimentation coefficient	*s.*
sedimentation coefficient in water at 20°, extrapolated to zero concentration	$s^0_{20,w}$
Svedberg unit of sedimentation coefficient (10^{-13} sec.)	S
diffusion coefficient (usually given in $cm.^2$/sec.)	*D*

Spectrophotometric Units

A = Absorbance = $\log_{10}(1/T) = \log_{10}(I_0/I)$ where T = transmittance, I_0 = intensity of radiation entering the medium, I = intensity after traversing the medium (The term "absorbance" is preferred to "optical density.")

ϵ = molecular extinction coefficient = AM/bc where M is molecular weight, b is cell length in centimeters, and c. the concentration in grams per l.

Equilibrium and Velocity Constants

Dissociation constants, association constants, and Michaelis constants should ordinarily be written in terms of concentrations in moles per liter; for instance, for the reaction $Mg^{++} + ATP^{4-} \rightleftharpoons MgATP^{2-}$, the association constant is: $K = (MgATP^{2-})/(Mg^{++})(ATP^{4-})$; (in units of M^{-1}).

If other units of concentration are employed, they should be clearly indicated at the point where the equilibrium constant is defined, and where its value is given.

Values of velocity constants should be similarly specified, first order velocity constants being generally given in $sec.^{-1}$ (other units of time may be used on occasion, but in any case the time unit should be specified). Second order velocity constants are ordinarily given in M^{-1} $sec.^{-1}$.

The term milligram per cent (mg.%) should not be used. Weight concentrations should be given as gm. per ml., gm. per 100 ml., gm. per l., etc.

Studies of Ribose Metabolism

VII. AN ASSESSMENT OF RIBOSE BIOSYNTHESIS FROM HEXOSE BY WAY OF THE C-6 OXIDATION PATHWAY*

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(Received for publication, May 26, 1958)

Reactions have recently been described which mediate the oxidative removal of carbon 6 of D-glucuronic acid and the conversion of the product to ribose phosphate (1–6). Observations indicating the operation *in vivo* of this C-6 oxidation pathway in man (7) have prompted us to investigate its role in ribose biosynthesis from hexose. Previous studies of ribose production from glucose-C^{14} have been interpreted as demonstrating that the pentose is derived directly via the oxidative and nonoxidative reactions of the pentose phosphate pathway (8–10). The substantial quantity of ribose-2-C^{14} produced by human subjects (11) and by rats (9, 10) given glucose-2-C^{14} was considered to have been synthesized by way of the reactions catalyzed by transketolase and transaldolase. It is apparent, however, that pentose arising from the oxidative removal of carbon atom 6 of hexose-2-C^{14} would also be labeled in its second position. Hence, studies with glucose-2-C^{14} cannot distinguish between pentose derived from the transketolase-transaldolase reactions, on the one hand, and the C-6 oxidation pathway, on the other. Such a differentiation can be made, however, by examining the isotope pattern in ribose synthesized from glucose-3,4-C^{14}. Pentose arising from such hexose by way of the oxidative removal of carbon atom 6 should be labeled equally in Positions 3 and 4. In marked contrast, ribose synthesized from glucose-3,4-C^{14} via the transketolase-transaldolase series of reactions would be labeled in carbon atoms 1, 2, and 3, but not in 4 (12). Finally, ribose synthesized from hexose-3,4-C^{14} via the oxidative reactions of the pentose phosphate pathway catalyzed by glucose-6-phosphate and 6-phosphogluconic dehydrogenases would be labeled in carbon atoms 2 and 3.

In the studies to be reported the incorporation of glucuronolactone carbon into ribose was observed in the rat. Despite this presumptive evidence for the operation of the C-6 oxidation pathway, the isotope pattern in ribose synthesized from glucose-3,4-C^{14} indicates that little if any ribose is derived from hexose by way of glucuronic acid. This situation prevailed even in rats treated with Chloretone (Parke, Davis and Company), which is known to stimulate at least some of the reactions of the C-6 oxidation pathway (13, 14).

EXPERIMENTAL

To obtain ribose for these studies, advantage was taken of the findings of Tabor and Hayaishi (15) and of Karjala (16) that imidazoleacetic acid riboside is excreted in the urine after the administration of imidazoleacetic acid. Ribose was isolated from urinary imidazoleacetic acid riboside of 250 gm. male Sprague-Dawley rats which had received intraperitoneal injections of imidazoleacetic acid hydrochloride and D-glucuronolactone uniformly labeled with C^{14} (glucuronolactone-U-C^{14}) or glucose-3,4-C^{14}, according to procedures described in detail in an earlier report (10). The ribose degradation procedure and the analytical methods involved are summarized elsewhere (17). The animals were permitted access to Purina chow until the time of administration of the acid but were fasted throughout the period of urine collection. For 8 days before study the Chloretone-stimulated rats of Experiment 28 were fed ground Purina chow to which had been added 6 mg. of Chloretone per gm. of diet. At the time of the study the animals were somnolent and showed the anticipated rise (13, 14) in urinary ascorbic acid from a control level of 0.2 mg. to a final level of 6.2 mg. per 24 hours For 10 days the rats in Experiment 35 were fed by tube with 1 ml. of evaporated milk containing 40 mg. of Chloretone. Urinary ascorbic acid was measured by the procedure of Roe and Kuether (18).

Radioactive substrates—D-glucuronolactone uniformly labeled with C^{14} was a generous gift of Dr. N. E. Artz of the Corn Products Refining Company, Argo, Illinois.

Glucose-3,4-C^{14} was prepared by administering $NaHC^{14}O_3$ intraperitoneally and glucose by gavage to rats which had been fasted for 48 hours, and then by isolating (19) and hydrolyzing (20) liver glycogen. The distribution of C^{14} in the glycogen glucose molecule was determined according to procedures described elsewhere (21) and was found to be as follows: Carbon 1, 0.9 per cent; Carbon 2, 0.5 per cent; Carbon 3, 48.2 per cent; Carbon 4, 50.4 per cent; and Carbons 5 and 6, 0 per cent.

RESULTS

The quantity of ribose excreted in the urine as imidazoleacetic acid riboside and the proportion of the administered C^{14} incorporated in the ribose of animals which had been given injections of glucuronolactone or glucose are presented in Table I. The isotope distribution in the ribose of the animals given glucose 3,4-C^{14} is depicted in Table II. In both the normal and the Chloretone-stimulated animals the ribose was predominantly labeled in carbon atoms 1, 2, and 3, and little radioactivity appeared in Carbon 4 relative to Carbon 3.

* This investigation was aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research and by Grant No. C-3151 from the United States Public Health Service.

TABLE I

Urinary ribose of rats given imidazoleacetic acid

Experiment No.	Animal status	ImAA* administered	Urinary ribose	C^{14} compound administered	Molar activity of ribose	C^{14} in ribose	Administered C^{14} in ribose
		μmoles	*μmoles*		*c.p.m./μmole*	*c.p.m.*	%
13	Normal	500	155	Glucuronolactone-U-C^{14} (5.39×10^6 c.p.m.; 0.13 mmole)	126	19,500	0.36
25	Normal	500	99	Glucose-3,4-C^{14} (2.57×10^6 c.p.m.; 1.03 mmole)	135	13,400	0.52
28	Chloretone-stimulated	500	123	Glucose-3,4-C^{14} (1.85×10^6 c.p.m.; 0.74 mmole)	119	14,600	0.79
35	Chloretone-stimulated	500	91	Glucose-3,4-C^{14} (1.24×10^6 c.p.m.; 0.50 mmole)	58	5,300	0.43

* ImAA = imidazoleacetic acid.

TABLE II

Isotope distribution in urinary ribose of rats given imidazoleacetic acid and glucose-3,4-C^{14}

Experiment No.	Animal status	Relative activity in ribose carbon atoms*				
		C-1	C-2	C-3	C-4	C-5
25	Normal	39	67	100 (1600)	9	6
28	Chloretone-stimulated	36	73	100 (2040)	16	7
35	Chloretone-stimulated	42		100 (2240)	10	6

* The specific activity of C-3, which is assigned an arbitrary value of 100, is indicated by the figure in parentheses (c.p.m. per mmole of carbon).

DISCUSSION

We have recently described the incorporation of substantial radioactivity into ribose excreted by a normal human subject given imidazoleacetic acid and glucuronolactone-U-C^{14} and the virtual absence of isotope in the urinary ribose of an individual with the genetic disturbance, chronic essential pentosuria (7). Since a block in the C-6 oxidation pathway is believed to be the metabolic aberration in pentosuria (22), our studies contribute evidence for the operation of this pathway in ribose biosynthesis from glucuronic acid in normal man. Presumably, the same mechanism accounts for the appearance of C^{14} in the urinary ribose of the rat given glucuronolactone-U-C^{14} in the present study. Although endogenous glucuronic acid is synthesized from glucose (23), the demonstrated capacity of the organism to convert administered glucuronic acid carbon to ribose does not, of course, imply that the acid is a normal intermediate in ribose biosynthesis from hexose. Indeed, the role of this pathway in ribose biosynthesis from hexose appears to be relatively minor, as determined by the labeling pattern in ribose synthesized from glucose-3,4-C^{14}. These results, which are similar to the isotope distribution observed in nucleic acid ribose isolated by Bernstein from rats given $C^{14}O_2$ (24), provide support for the hypothesis that ribose is synthesized by the C-1 oxidation and the transketolase-transaldolase mechanisms and exclude substantial ribose production from hexose by way of C-6 oxidation. Since Chloretone is known to increase glucuronic acid (14) and ascorbic acid (13) excretion, indicating a stimulation of at least some of the reactions in the C-6 oxidation pathway, it is of interest that rats treated with this drug showed no increase in ribose synthesis from hexose via glucuronic acid.

SUMMARY

Ribose biosynthesis from D-glucuronic acid has been demonstrated in the rat. The pathway involved is presumably that involving oxidative removal of Carbon 6 of the uronic acid. Studies of the isotope pattern in ribose synthesized from glucose-3,4-C^{14} indicate that this mechanism plays little if any role in ribose production from hexose in untreated and in Chloretone-stimulated rats.

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Requirements of Mn^{++} and Co^{++} for the Synthesis of Ascorbic Acid by Liver Extracts of Animals Deprived of Tocopherol*

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(Received for publication, June 2, 1958)

It has been found, by Dam *et al.* (1), that supplements of ascorbic acid can prevent the development of encephalomalacia or exudative diathesis in chickens when they are fed diets that are deficient in tocopherol. Current experiments in this laboratory indicate that the presence of ascorbic acid in certain diets that are low in tocopherol content results in delayed onset of muscular dystrophy in rabbits. One possible explanation of the foregoing is that the rate of ascorbic acid synthesis is impaired in vitamin E deficiency.

The work of ul Hassan and Lehninger (2) and Grollman and Lehninger (3) on the synthesis *in vitro* of ascorbic acid by animal tissues provided a means to compare the rates of synthesis by vitamin E-deficient animals and their controls. This communication contains the results of such a study which revealed that this synthesis is dependent on coenzymatic factors altered by the deficiency *in vivo* of vitamin E.

EXPERIMENTAL

Animals—Male rats, weighing 100 to 150 gm. were taken from our colony of albino rats developed from Holtzman's Sprague-Dawley strain (Madison, Wisconsin). Male New Zealand white rabbits weighing 500 to 700 gm. were obtained from the National Animal Laboratories, Creve Coeur, Missouri.

Diets—The compositions of the diets (Table I) are modifications of a diet developed by Young and Dinning (4). These diets produced sterility in rats and muscular dystrophy in rabbits, with the exception of the basal diet plus vitamins A and D which caused dystrophy only occasionally. The deficiency symptoms could be prevented by oral administration three times per week of 50 mg. of α-tocopheryl acetate to rabbits and 10 mg. to rats. The animals were fed *ad libitum*, and food consumption by the deficient and control groups was approximately the same, averaging 40 to 45 gm. per day for rabbits and 10 to 18 gm. per day for rats. Weight gain differences between the two groups were not significant for the period of the experiments. Distilled water was provided.

Enzyme Preparation—2 to 20 days after initiation of the diets, the control and experimental rats or rabbits were killed by a blow on the head and then bled. Livers were removed, placed in cold 0.15 M potassium phosphate buffer, pH 7.5, and quickly weighed to prepare 20 per cent weight per volume homogenates in fresh buffer using all glass homogenizers. The homogenates were centrifuged for 10 minutes at 5200 × *g* (4°) to remove blood cells, nuclei, and mitochondria. The supernatant solution, containing soluble enzymes and microsomes, was designated the enzyme preparation. For certain experiments requiring further fractionation this extract was centrifuged at 100,000 × *g* for 30 minutes at 4° in the Spinco model L ultracentrifuge. Microsomes were resuspended at their original concentration in 0.15 M phosphate buffer.

Test System—The incubation system was that of ul Hassan and Lehninger (2) with modifications. Grollman and Lehninger (3) showed that TPN is a cofactor in the step D-glucuronate → L-gulonate. In our experience, the addition of ATP to the reaction instead of TPN resulted in a 20 to 50 per cent higher rate of synthesis, which suggests that TPN is maintained at higher levels during the incubation when it is supplied through the reaction ATP + DPN → ADP + TPN than when it is added at the beginning of the reaction. The test system contained the following: ATP, 2.3 μmoles; DPN, 1.8 μmoles; phosphate buffer, pH 7.5, 30.0 μmoles; nicotinamide, 20.0 μmoles; glucuronic acid (neutralized with NaOH), 10.0 μmoles; and 0.25 ml. of the enzyme preparation. Total volume was 0.8 ml., except when specified additions were made. Incubation was at 37° for 2 hours; the reaction was terminated by adding 2.5 ml. of 6 per cent trichloroacetic acid. Initial ascorbic acid was determined by an identical system, except that glucuronate was added after inactivation. Ascorbic acid was measured by the 2,4-dinitrophenylhydrazine method of Roe and Kuether (5), with the modifications of Geschwind *et al.* (6). As is shown by ul Hassan and Lehninger (2), the main product of the test system determined by this method is L-ascorbic acid. Occasionally the 2,6-dichlorophenolindophenol method described by Roe (7) was used, and the results were in agreement with the Roe and Kuether method. The relationship between the amount of enzyme preparation of the control rat liver in the test system and the formation of ascorbic acid is shown in Fig. 1.

Determination of Metals

Manganese—The periodate method was used. 1 gm. of liver was ashed overnight in a 30-ml. Kjeldahl flask with 4 ml. of a mixture of concentrated HNO_3, 30 per cent perchloric acid, and 9 N H_2SO_4 (1:2:1). The clear digest was transferred to a 4-ml. graduated tube with N H_3PO_4 and 2 mg. of KIO_4 added. The

* Supported by research grants from the National Institutes of Health, United States Public Health Service (Nos. A-1174 and A-860) and the Williams-Waterman Fund for the Combat of Dietary Diseases.

TABLE I

Tocopherol-deficient diets used

The basal diet (grams) consisted of: casein,* 15; sucrose, 39; corn starch, 36; salt mixture,† 3. The vitamins* per 100 gm. of diet were: nicotinamide, 20 mg.; pyridoxine-HCl, 0.5 mg.; thiamine-HCl, 0.5 mg.; riboflavin, 0.5 mg.; calcium pantothenate, 1 mg.; folic acid, 0.5 mg.; biotin, 0.005 mg.; 2-methylnaphthoquinone, 0.025 mg.; vitamin B_{12}, 4.5 μg.; choline chloride, 0.1 gm.; inositol, 0.1 gm. The vitamins were triturated in dextrose. 10 gm. of powdered cellulose (Alphacel)* were added per 90 gm. of diet after the additions as indicated:

Name of diet	Additions to basal diet
BD + A + D‡.....	Lard,* 6 gm.; A, 10 mg.; D, 0.6 mg.
BD + 1.5% CLO.....	Lard, 4.5 gm.; CLO, 1.5 gm.
BD + 3.0% CLO.....	Lard, 3 gm.; CLO, 3 gm.
BD + 1.5% CLO + A + D.....	Lard, 4.5 gm.; CLO, 1.5 gm.; A, 10 mg.; D, 0.6 mg.
BD + 1.5% CLO + A + D + C.....	Lard, 4.5 gm.; CLO, 1.5 gm.; A, 10 mg.; D, 0.6 mg.; C, 100 mg.

* Casein, vitamins, salt mixture, cod liver oil, and Alphacel were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Molecularly distilled lard was from Distillation Products Industries, Rochester, N. Y.

† Hubbell, *et al.* (8).

‡ BD, basal diet; A, C, and D correspond to vitamin supplement to diet; CLO, cod liver oil supplement to diet.

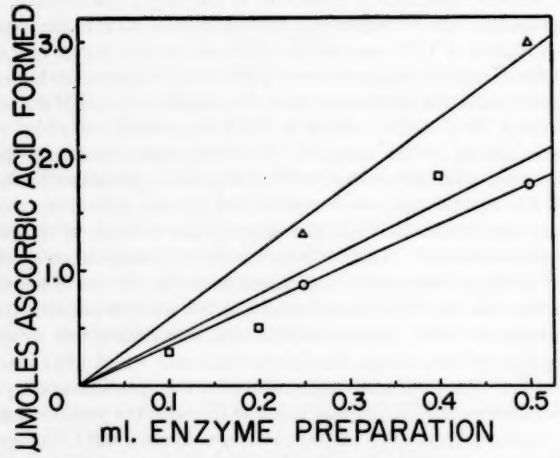

FIG. 1. Relationship between amount of enzyme preparation and ascorbic acid synthesis for three different preparations. The methods are as in "Experimental."

mixture was then immersed 15 minutes in a boiling water bath, cooled, and read at 525 mμ in the Beckman model DU spectrophotometer.

Calcium—The liver homogenates were deproteinized with 5 per cent (end concentration) of trichloroacetic acid. The supernatant solution was then adjusted to pH 5.0 with N NH_4OH and was titrated with EDTA[1] with the use of calcein (obtained from G. Frederick Smith Chemical Company, Columbus, Ohio) as indicator (9).

Magnesium—This was determined in the supernatant layer after Ca^{++} was precipitated with ammonium oxalate. The solution was made alkaline with concentrated NH_4OH and the Mg^{++} precipitated overnight at room temperature after addition of KH_2PO_4. The precipitate was centrifuged, washed twice with a mixture of NH_4OH, ethanol, and water (1:4:5), and dried at 100° in an oven. The ammonium magnesium phosphate was dissolved in dilute H_2SO_4, and the phosphate was determined by the method of Fiske and SubbaRow.

Iron—Total iron was measured with 4,7-diphenyl-1,10-phenanthroline according to the method of Peterson (10). Ferrous iron was estimated by a modification in which thioglycolic acid was omitted from the hot trichloroacetic acid treatment.

[1] The abbreviation used is: EDTA, ethylenediaminetetraacetate.

RESULTS

Impairment of Ascorbic Acid Synthesis in Deficient Animals—Experiments that measure formation of ascorbic acid are summarized in Table II. There is a 70 to 90 per cent decrease in the rate of synthesis by enzyme preparations of tocopherol-deficient rats of the different dietary groups. Similar results were obtained with a group of rabbits. It is generally accepted that cod liver oil is a necessary factor in diets to produce muscular dystrophy in rabbits. From Table II it is apparent, however, that cod liver oil is not required for the development of defective ascorbic acid synthesis in the rat even if there is a trend toward aggravation of the effect with increasing amounts of the oil. An inter-

TABLE II

Rate of ascorbic acid synthesis and destruction by liver preparations from animals on different diets

For conditions and methods, see "Experimental." In destruction experiments, glucuronate was replaced by 25 μg. of L-ascorbate. Names of diets refer to Table I.

Animal	Name of diet	No. pairs of animals	Supplement to diet	Ascorbic acid synthesis
				*μmole/gm. wet tissue/2 hrs.**
Rat	BD + A + D†	14	None	0.52 ± 0.14
			Vitamin E	1.62 ± 0.25
	BD + 1.5% CLO + A + D	13	None	0.29 ± 0.07
			Vitamin E	1.37 ± 0.19
	BD + 3.0% CLO	9	None	0.26 ± 0.08
			Vitamin E	1.00 ± 0.12
	BD + 1.5% CLO + A + D + C	11	None	0.18 ± 0.07
			Vitamin E	2.38 ± 0.19
Rabbit	BD + 3.0% CLO	8	None	0.31 ± 0.10
			Vitamin E	1.47 ± 0.26
				Ascorbic acid destruction
Rat	BD + 1.5% CLO + A + D	4	None	1.37 ± 0.10
			Vitamin E	1.25 ± 0.12

* Mean ± standard error.

† BD, basal diet; A, C, and D correspond to vitamin supplement to diet; CLO, cod liver oil supplement to diet.

esting point is that the presence of ascorbic acid in the diet did not increase synthesis by vitamin E-deficient animals. This indicates that those factors involved in the production of encephalomalacia and exudative diathesis in the chick, which were prevented by ascorbic acid (1), are not the same as those that impair ascorbic acid synthesis.

The possibility was considered that the difference in the rates of synthesis was only apparent and due to converse differences in the rates of destruction. This was discarded when an investigation of destruction of ascorbic acid by the enzyme preparations of control and deficient animals showed no differences (Table II). In the destruction experiments the conditions were identical to those of synthesis, but glucuronate was replaced by L-ascorbic acid.

Development and Duration of Effect—The condition developed rapidly when the animals were deprived of vitamin E. The control group attained highest rates of ascorbic acid synthesis when vitamin E was given 12 to 16 hours before the group was killed. Data on the development of the effect showed that the synthesis reached the lowest levels after 3 or 4 days and did not change up to 20 days when the experiments were terminated. Oral administration of α-tocopheryl acetate not only prevented but also reversed the effect.

Efforts to Localize Biochemical Disorder—The enzymes involved in the synthesis of ascorbic acid by rat liver are distributed between the soluble and the microsome fractions (2). These two fractions were separated and recombined to put together the microsomes from deficient animals with the supernatant fraction of the controls and *vice versa*. Thirteen experiments of this type were performed. Nine of the combinations of the "control" microsomes with the "deficient" supernatant solution reached, partially or fully, the level of synthesis by preparations from the control animals. This would point to the "deficient" microsomes as being the defective fraction. However, in the combinations of "deficient" microsomes with "control" supernatants, there was a substantial synthesis in six cases, although not as much as in the other combination. The provisional interpretation of these experiments is that the primary cause of the impairment is not a decrease of the enzymes of either the supernatant fraction or the microsomes since both fractions could be reactivated by changing the conditions under which they are incubated.

Experiments to elucidate whether or not the heat-stable fraction of control preparations reactivates the extracts from deficient animals gave equivocal results. In experiments testing for the presence of a toxic factor in livers of deficient rats, the results were also equivocal.

Attempts to reactivate with tocopherol were negative when the vitamin was added to the systems without a solubilizing agent. When such agents (alcohol, ether, and albumin) were used, no conclusion could be drawn because of the inactivating effect they exerted on the reaction.

Action of Metal Ions—The activation or inhibition by metal ions of synthesis by the enzyme preparations from both control and deficient animals offers a possible explanation of these findings. The effects of various metal ions on ascorbic acid synthesis were consistent and independent of the type of tocopherol-deficient diet fed and, therefore, the data for animals in the different dietary groups were pooled in Table III.

Mg^{++} has been shown to be a cofactor for the system (2). The undialyzed control of our experiments had no Mg^{++} requirements which indicated that the extraction of this ion from liver tissue was sufficient for the reaction. Synthesis by preparations from deficient animals was not activated by concentrations of Mg^{++} from 3×10^{-6} to 3×10^{-2} M.

TABLE III

Effect of metal ions on synthesis of ascorbic acid by enzyme preparations from livers of rats on vitamin E-deficient and control diets

The methods are as in "Experimental." The end concentration of $MgSO_4$ is 5×10^{-2} M; of other salts, 5×10^{-4} M.

Addition to test system	No. pairs of rats	Supplement to diet*	Ascorbic acid synthesis	
			No metal added	Metal added
			μmole/gm. wet tissue/2 hrs.†	
$MgSO_4$	22	None	0.40 ± 0.24	0.37 ± 0.08
		Vitamin E	1.62 ± 0.25	1.62 ± 0.24
$CaCl_2$	19	None	0.41 ± 0.08	0.28 ± 0.08
		Vitamin E	1.36 ± 0.15	1.12 ± 0.19
$FeSO_4$	6	None	0.23 ± 0.07	0.26 ± 0.07
		Vitamin E	1.64 ± 0.41	1.03 ± 0.13
$FeCl_2$	4	None	0.13 ± 0.07	0.18 ± 0.12
		Vitamin E	2.16 ± 0.47	1.39 ± 0.33
$MnSO_4$ or $MnCl_2$	34	None	0.33 ± 0.06	1.37 ± 0.15
		Vitamin E	1.57 ± 0.20	1.35 ± 0.15
$CoCl_2$	26	None	0.31 ± 0.05	1.17 ± 0.16
		Vitamin E	1.77 ± 0.17	2.39 ± 0.20

* For the diets used see under "Action of Metal Ions."
† Mean ± standard error.

The extracts of control animals also had no requirement for Mn^{++} but, when the ion was added at a concentration of 5×10^{-4} M to the test system for deficient animals, the formation of ascorbic acid was activated to the control level (315 per cent increase). Analyses described later in this report disclosed no decrease in total Mn^{++} content of liver in the deficient rats. This suggested that an abnormal mineral metabolism may have developed and, to test this hypothesis, the effect of other cations was assayed.

Ca^{++} (5×10^{-4} M) did not activate synthesis by extracts from deficient animals. Its addition to the control system decreased ascorbic acid formation by 18 per cent.

The importance of Fe^{++} and Fe^{+++} as factors in the production of mouse paralysis due to vitamin E deficiency has been demonstrated by King *et al.* (11). They suggested that the effects of these metal ions may be modified by other trace elements. Neither Fe^{++} nor Fe^{+++} produced a significant activation of synthesis by deficient rats, but both inhibited the activity of the controls by approximately 35 per cent.

The addition of 5×10^{-4} M Co^{++} produced an increase of 278 per cent in the rate of ascorbic acid formation by deficient animal liver extracts. Co^{++} was the only metal of those tested which increased synthesis by control preparations (35 per cent increase). The effects produced by Mn^{++} and Co^{++} in enzyme preparations of deficient animals were not additive.

Other salts tested were $ZnSO_4$ (3 experiments), $CdCl_2$, (1 experiment), and LiCl (3 experiments) at 5×10^{-4} M concentration. These either had no effect or produced some inhibition of ascorbic acid synthesis in both groups of animals.

Determinations of Metals in Liver and Enzyme Preparations—Since the addition of Mn^{++} to liver extracts of tocopherol-deficient animals resulted in a restoration of synthesis, this metal

TABLE IV

Comparison between metal contents of livers of tocopherol-deficient and control rats and their rates of ascorbic acid synthesis

Metal	No. of Experiments	Supplement to diet*	Ascorbic acid synthesis	Metal concentration	
				Whole liver	Enzyme preparation
			μmole/gm. wet tissue/2 hrs.†	*μg./gm. wet tissue*‡	
Mn	5	None	0.28	1.8	1.1
		Vitamin E	1.28	1.7	1.1
Ca	3	None	0.31	36.0	
		Vitamin E	1.78	37.0	
Mg	3	None	0.09	149.0	129.0
		Vitamin E	2.55	150.0	129.0
Fe	3	None	0.76		136.0
		Vitamin E	1.90		173.0
Fe^{++}	3	None	0.76		47.0
		Vitamin E	1.90		79.0

* Diet: basal diet plus 1.5% cod liver oil.
† Mean values.
‡ For methods see "Experimental."

was determined in the liver tissue of these animals and their controls. No difference in either total Mn^{++} or that of the enzyme preparation fractions was observed (Table IV).

The determination of Ca^{++} in the whole livers showed no difference between the deficient and control groups. Mg^{++}, which was assayed both in whole livers and in the enzyme preparations, also failed to show differences. The measurement of iron showed slightly less total Fe in the deficient animals. Fe^{++}, as determined by the procedure described under "Determination of Metals," was also observed to be lower in that group.

Activating Effect of EDTA—The demonstration that there was no measureable difference in the Mn^{++} content of the liver tissue of the deficient and control animals, together with the finding that the addition of this metal produced activation only in the deficient animals, suggested that availability of tissue Mn^{++} is altered in vitamin E deficiency as the result of an imbalance of an unknown nature. This hypothesis is supported by the results of experiments with chelating agents. When EDTA is introduced into the test system at concentrations from 6×10^{-5} to 6×10^{-4} M, the synthesis of ascorbic acid by preparations from deficient animals is 480 per cent of that without EDTA, whereas the value for the controls with the same additions decreased slightly (Table V). Similar results were obtained in 3 experiments with diphenylthiocarbazone (dithizone) and in one experiment with 1-nitroso-2-naphthol-3,6-disulfonic acid, disodium salt. Other metal complex-forming substances tested were NaF (6 experiments) and KCN (1 experiment). These had no influence on the formation of ascorbic acid by liver extracts from either group of animals. In one experiment, cysteine (6×10^{-5} M) was ineffectual as an activator and resulted in decreased synthesis by the control.

DISCUSSION

These results have disclosed an impairment of ascorbic acid synthesis by extracts of livers from rabbits and rats deprived of vitamin E. It is not possible at present to determine the importance of this impairment as a mechanism for the production of the symptoms of vitamin E deficiency. In more than 75 deficient rats which were used in these experiments, the average synthesis of ascorbic acid was approximately 0.2 μmoles per gm. per 2 hours, which is equivalent to more than 2 mg. of ascorbic acid per day for a rat with a 5-gm. liver. The guinea pig is the only small laboratory animal for which the minimal requirement of ascorbic acid is known, and this has been determined to be 0.5 mg. per day (12). If these figures can be used to estimate the requirement for the rat, it is unlikely that the impaired production of ascorbic acid is responsible for symptoms of vitamin E deficiency in the rat.

An outcome of this work is the correlation of tocopherol with the function of several metals as coenzymatic factors. This should be referred to the reports of King *et al.* (11) and Lee *et al.* (13) according to which the mineral content of the diet determines the character of the manifestations of vitamin E deficiency. An understanding of the mechanism of this interaction will have to wait until more is known about the action of metals, especially Mn^{++} and Co^{++}. The findings presented do not support the explanation that deficiency of vitamin E produces a general or specific metal deficiency in the tissues, since the manganese content of the livers of vitamin E-deficient rats is not lowered. Furthermore, the impairment in synthesis was also corrected by agents that form metal complexes. An alternative interpretation

TABLE V

Effect of EDTA and of dithizone on synthesis of ascorbic acid by livers from rats on vitamin E-deficient and control diets

For methods see "Experimental" section.

Addition to test system	Final concentration	No. pairs of rats	Supplement to diet*	Ascorbic acid synthesis	
				No additions	Complex-forming agent added
				μmole/gm. wet tissue/2 hrs.†	
EDTA	6×10^{-5} M	15	None	0.25 ± 0.06	1.20 ± 0.08
			Vitamin E	1.77 ± 0.21	1.27 ± 0.23
EDTA	6×10^{-4} M	8	None	0.32 ± 0.03	1.35 ± 0.16
			Vitamin E	1.47 ± 0.44	1.12 ± 0.21
Dithizone	0.09 saturation	3	None	0.18 ± 0.13	0.59 ± 0.26
			Vitamin E	2.05 ± 0.36	1.32 ± 0.61

* Diet: basal diet, plus 1.5% cod liver oil, plus vitamins A, D and C.
† Mean ± standard error.

is that the accumulation of some other metals produced an imbalance which can be corrected by an excess of the activating metal or by a chelating agent. The determination of Ca^{++}, Mg^{++}, and total Fe did not support this hypothesis since they are not increased in the deficient tissues. A hypothetical mechanism that conforms to the data is that, in the absence of tocopherol, the states of some metals are altered to make them less efficient in their function or even to produce toxic reactions. The reducing properties of tocopherol suggest the possibility of changes in the relative amounts of the oxidized and reduced states of certain metals. The ability of EDTA and of other complex-forming agents to restore ascorbic acid synthesis could be related to the displacement of oxidation-reduction equilibria through preferential complex formation with a component of the system. Belcher *et al.* (14) reported that the systems Fe^{++}-Fe^{+++} and Cu^{+}-Cu^{++} are displaced toward a prevalence of the reduced states in the presence of EDTA.

A relevant question is whether the alteration of the function of metals is directly or indirectly related to the deficiency of tocopherol. The early appearance of the impairment of synthesis of ascorbic acid, compared for instance with the onset of creatinuria (15), suggests that the relationship is direct.

SUMMARY

Enzyme preparations from livers of vitamin E-deficient rats and rabbits synthesized 70 to 90 per cent less ascorbic acid than preparations from control animals. The effect was observed after feeding diets with a low content of tocopherol from 2 to 20 days at which time the experiments were terminated. Addition of Mn^{++} increased synthesis 315 per cent in preparations from deficient animals but had no effect on preparations from controls. Co^{++} produced increases of 278 per cent and 35 per cent in deficient and control preparations, respectively. Ethylenediaminetetraacetate activated the preparations from deficient animals 480 per cent but had no effect on control animal systems.

Determinations of Mn^{++}, Ca^{++}, and Mg^{++} in the livers disclosed no differences between control and deficient animals. Total Fe and Fe^{++} ions were slightly diminished. The findings suggested that in tocopherol deficiency the states of some metal ions are altered, causing them to function less efficiently.

Acknowledgments—We are indebted to Distillation Products Industries, Rochester, New York, and the Sigma Chemical Company, St. Louis, Missouri, for providing part of the materials used in this work.

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The Metabolism of Mucopolysaccharides in Mammalian Tissues

V. THE ORIGIN OF L-IDURONIC ACID*

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(Received for publication, June 26, 1958)

L-Iditol was first isolated from the mountain ash berry by Bertrand in 1905 (1, 2). No further indication of the presence of L-idose derivatives in nature occurred until the reports of Hoffman *et al.* (3) and Cifonelli *et al.* (4) which indicated that the acid mucopolysaccharide, CSA-B[1] (β-heparin), contains iduronic acid. The latter workers have established the configuration as L and have isolated a disaccharide which appears to be 1,3-iduronosylgalactosamine (4). Since methylation studies by Jeanloz *et al.* (5) indicated that the sulfate group cannot be on position 6 of the galactosamine moiety, it was pointed out that it must be linked to position 4, a suggestion which has received support from the infrared studies of Mathews (6). Jeanloz and Stoffyn (7) have recently confirmed the 3-glycoside linkage by methylation studies and the configuration of the L-iduronic acid. The identity of L-iduronic acid has been established by conversion to idose, iditol, and idonic acid.

The presence of L-iduronic acid in CSA-B contrasts with the presence of D-glucuronic acid in hyaluronic acid, CSA-A, and heparin. Earlier studies have established that in streptococci both the glucuronic acid (8) and glucosamine moieties (9, 10) of hyaluronic acid are derived from glucose without fragmentation of the carbon chain. Direct evidence for the origin of the glucuronic acid from glucose in mammalian mucopolysaccharides so far has not been obtained. However, the origin of the glucuronic acid of glucuronides from glucose without scission of the chain is firmly established both by virtue of studies *in vivo* (11–13) and by the elucidation of the enzymatic mechanisms (14–16).

It is the purpose of this communication to present evidence which shows that both the L-iduronic acid of CSA-B and the D-glucuronic acid of hyaluronic acid of skin are derived from glucose without splitting of the carbon chain.

EXPERIMENTAL AND RESULTS

Each of a group of 30 white Sprague-Dawley rats with a mean weight of 210 gm. was given an injection of 19.2 mg. of glucose-6-C^{14} (specific activity 2.32 μc. per mg.) in an isotonic solution. 26 hours after injection the rats were killed and hyaluronic acid and CSA were prepared as previously described (17). The nitrogen-hexosamine ratios of both polysaccharides were 1.6:1.7 after slab electrophoresis. This indicated the presence of small amounts of protein impurities, and the material was therefore further purified by means of precipitation with cetyl pyridinium chloride according to Scott (18, 19). 42 ml. of a hyaluronic acid solution which contained approximately 1 mg. per ml. were precipitated with 22 ml. of 1 per cent cetyl pyridinium chloride in the presence of 0.004 M Na_2SO_4. After incubation at 37° for 6 hours the precipitated cetyl pyridinium mucopolysaccharide complex was collected by centrifugation, dissolved in 10 ml. of 1.25 M NaCl, and the polysaccharide was precipitated by the addition of 40 ml. of 95 per cent ethanol. After 3 hours at 4°, the precipitate was collected by centrifugation, washed with ethanol and ether, and dried in a desiccator over P_2O_5. The CSA fraction was purified in a similar manner except that the cetyl pyridinium complex was dissolved in 1.5 M NaCl. Analyses of the products showed ratios of nitrogen to hexosamine to uronic acid of 1.09:1.00:1.01 for hyaluronic acid and 0.92:1.00:0.48 for CSA; glucuronolactone was used as a standard for the uronic acid determination.

Isolation of CSA-B—Earlier studies from this laboratory have demonstrated that the CSA fraction isolated from rat and rabbit skin is hydrolyzed by testicular hyaluronidase to the extent of 30 to 40 per cent (17). Since CSA-B is not attacked by testicular hyaluronidase, these findings have been interpreted as an indication of the presence of CSA-A or CSA-C.

In order to study the metabolic origin of L-iduronic acid it was necessary, therefore, to free CSA-B from CSA-A or CSA-C. Two criteria were used to establish the purity of the isolated CSA-B. The first method depended upon susceptibility to highly purified testicular hyaluronidase. The second method utilized the colorimetric reactions of the iduronic acid and glucuronic acid. Since both glucuronic acid and iduronic acid give similar color equivalents by the orcinol method (20), but different equivalents in the carbazole reaction (21), the ratio of the extinction coefficients obtained by these two methods is a sensitive indicator of the nature of the uronic acid or the polysaccharides of which the two uronic acids are constituents (3). The carbazole to orcinol ratios for CSA-A (from cartilage), CSA-B, and glucuronolactone were found to be 1.2, 0.3, and 0.6 to 0.7, respectively.

It should be pointed out that the ratios given above were obtained with a Coleman Junior Spectrophotometer at 670 mμ and a heating time of 20 minutes as used by Brown (20). Hoff-

* This investigation was supported by grants from the National Heart Institute, United States Public Health Service (No. H-311), and the Chicago Heart Association.

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[1] The abbreviation used is: CSA, chondroitinsulfuric acid.

man *et al.* (3) have used a modification of this method with a heating time of 30 minutes.

When the content of CSA-B in the mixture isolated from skin was estimated on the basis of susceptibility to hyaluronidase and compared to the content estimated on the basis of the carbazole to orcinol ratio, a discrepancy was apparent. In the reaction with testicular hyaluronidase there was a 40 per cent disappearance of turbidity, which indicated the presence of 60 per cent CSA-B. However, the carbazole to orcinol ratio indicated a CSA-B content of 95 per cent. The reason for this difference is not yet clear. The possibility that the skin CSA contains a fraction hydrolyzed by testicular hyaluronidase, but containing iduronic acid, is at present under investigation.

Initially attempts to separate pure CSA-B were made by means of a modification of the procedure of Gardell (22), with alcohol fractionation on a cellulose column. Methanol and zinc acetate were substituted for the ethanol-barium acetate solvent since Marbet and Winterstein (23) previously demonstrated the difference in solubility of the zinc salts of CSA-A and CSA-B in 30 per cent methanol. When this method was applied to an artificial mixture of CSA-A isolated from cartilage and CSA-B (β-heparin) (kindly provided by Dr. A. Winterstein of Hoffmann-LaRoche, Inc., Basel, Switzerland), a satisfactory separation was readily achieved. When the same method was applied to mixed skin CSA, CSA-B could be isolated readily as was indicated by a carbazole to orcinol ratio of 0.24 for the fractions eluted by low methanol concentrations. (The fact that the value was lower than that obtained for the CSA-B from Dr. A. Winterstein is probably a result of the presence of another polysaccharide in the latter preparation.) At higher methanol concentrations a carbazole to orcinol ratio of 0.76 was obtained, which suggested a mixture of equal parts of polysaccharides containing iduronic and glucuronic acid.

A second method of purification was attempted in the hope that a more definitive separation could be achieved. This consisted of exhaustive digestion with testicular hyaluronidase of the mixed CSA fraction and then precipitation with cetyl pyridinium chloride. It was found that the products of hyaluronidase digestion of CSA-A or CSA-C were soluble in excess cetyl pyridinium chloride, although they could be precipitated by an equivalent amount of the detergent. In contrast, CSA-B is quantitatively precipitated in the presence of an excess of cetyl pyridinium chloride.

A model experiment with CSA-A and CSA-B is shown in Table I. It is evident that a complete separation has been achieved. It yielded a cetyl pyridinium chloride-insoluble CSA-B with a satisfactory carbazole to orcinol ratio and a cetyl pyridinium chloride-soluble fraction, which has the carbazole to orcinol ratio of CSA-A.

When this method was applied to skin CSA, a pure preparation of CSA-B was obtained which was not further digested by hyaluronidase. The carbazole to orcinol ratio was 0.22. The cetyl pyridinium chloride-soluble fraction, however, had a carbazole to orcinol ratio of 0.56. The possibility that the preparative method used for the skin mucopolysaccharides (17) gives CSA-B fractions soluble in cetyl pyridinium chloride was excluded by precipitation of skin CSA with excess cetyl pyridinium chloride and examination of the supernatant for uronic acid. All uronic acid was recovered in the precipitate. The finding that hyaluronidase releases material with a low carbazole to orcinol ratio agrees with the discrepancy between the enzymatic and colorimetric estimation of CSA-B content in skin.

TABLE I

Separation of CSA-A and CSA-B by digestion with hyaluronidase

The complete system contained: 5.0 mg. each of CSA-A and CSA-B in 1.0 ml. of H_2O; 7500 units of testicular hyaluronidase in 0.5 ml. of H_2O; and 0.5 ml. of 0.3 M NaH_2PO_4, pH 5.0. The final pH of the solution was 5.3. Incubation continued for 2 hours at 38°. To 1.0 ml. of the digest were added 2.0 ml. of 1 per cent cetyl pyridinium chloride. The precipitate was separated by centrifugation and washed with 1 per cent cetyl pyridinium chloride. After repeated centrifugation it was dissolved in 0.7 ml. of 1.25 M NaCl and precipitated with 2.1 ml. of ethanol. The precipitate was collected by centrifugation and washed once with ethanol and once with ether. The cetyl pyridinium chloride-soluble supernatant was filtered through a fine glass filter and passed through a 1 × 5 cm. column of Dowex 50, Na^+ (20 to 40 mesh). The column was subsequently washed with water. An identical procedure was followed for the skin sample except that skin CSA was substituted for the artificial mixture.

Material	Optical density ratios carbazole to orcinol
Individual compounds	
CSA-A (cartilage)	1.15
CSA-B (Winterstein)	0.33
Artificial mixture of CSA-A and CSA-B digested with hyaluronidase	
Cetyl pyridinium chloride-soluble fraction	1.25
Cetyl pyridinium chloride-insoluble fraction	0.33
CSA fraction of skin digested with hyaluronidase	
Cetyl pyridinium chloride-soluble fraction	0.56
Cetyl pyridinium chloride-insoluble fraction	0.22

The method involving hyaluronidase digestion and cetyl pyridinium precipitation was chosen for the isotope studies and was carried out in the following manner. 50 mg. of skin CSA were dissolved in 5.0 ml. of water and mixed with 1.0 ml. of 0.3 M NaCl and 2.0 ml. of 0.3 M NaH_2PO_4, pH 4.9. 3000 units of testicular hyaluronidase (activity 30,000 units per mg. of N) in 0.1 ml. of 0.02 M Na_2HPO_4, pH 7.0, were added, and the digestion was carried out for 24 hours with the addition of another 0.1-ml. portion of hyaluronidase after 8 hours. After digestion, 8.0 ml. of H_2O were added and the polysaccharide precipitated with 3.0 ml. of 1.0 per cent cetyl pyridinium chloride. The precipitate was collected by centrifugation and washed with 10 ml. of 1 per cent cetyl pyridinium chloride. The cetyl pyridinium complex was transformed to the sodium salt by dissolving in 4.0 ml. of 1.5 M NaCl and precipitating with 16.0 ml. of absolute ethanol. After 4 hours at 4° the precipitate was collected by centrifugation, washed with absolute ethanol and ether, and dried over P_2O_5. The yield was 28.5 mg. When this product was assayed for susceptibility to hyaluronidase by the turbidity method (24), approximately 10 per cent reduction of turbidity was obtained. However, when the digestion procedure was repeated, 25.2 mg. of a product completely resistant to hyaluronidase was obtained. As indicated in Table I, the carbazole to orcinol ration of this material was 0.22.

This procedure resulted in the isolation of apparently pure CSA-B which was used for degradations. The hyaluronic acid

TABLE II

Analyses of polysaccharide fractions

Preparation	Molar ratios*		
	Hexosamine†	Uronic acid‡	N§
"CSA fraction" of skin	1.00	0.48	0.92
Purified CSA-B (skin)	1.00	0.39	0.90
Hyaluronic acid	1.00	1.01	1.09

* Values are expressed as molar ratio with hexosamine taken as 1.00.

† Determined by the Boas modification (31) of the Elson-Morgan reaction with the resin treatment omitted.

‡ Determined by the Dische carbazole method (21).

§ Determined by a micro-Kjehldal procedure.

was obtained as previously outlined. The other CSA fraction was not characterized metabolically, since there was no proof that the supernatant after hyaluronidase digestion contained products derived from one polysaccharide. The analyses of the purified polysaccharide are shown in Table II. The appropriate degradations were carried out on the purified CSA-B fraction and hyaluronic acid. Radioactivity was determined in an internal gas flow counter. All samples were converted to $BaCO_3$ and corrected to infinite thinness. The C-6 of the uronic acid moiety was isolated after decarboxylation by refluxing in HCl, as previously described (9). The hexosamine was isolated from the hydrolysate by the method of Gardell (25). The acetyl group was isolated as the methyl benzimidazole according to Roseman (26).

Incorporation of Radioactivity—Table III shows the distribution of radioactivity in hyaluronic acid and CSA-B from skin subsequent to the injection of glucose-6-C^{14}. In the case of hyaluronic acid, the C-6 of glucuronic acid and glucosamine each contained 40 per cent of the total radioactivity. The acetyl moiety, which contains $\frac{2}{14}$ or 14 per cent of the carbon in the molecule, accounted for 5 per cent of the radioactivity. In the CSA-B, 43 per cent of the activity was present in the C-6 of iduronic acid; 43 per cent, in the galactosamine moiety; and 5 per cent, in the acetyl group. 84 and 91 per cent of the total radioactivity was accounted for in hyaluronic acid and CSA-B, respectively, by the parts of the molecule in which radioactivity was measured. It must be assumed that at least part of the remainder was contained in the C_{1-5} of the uronic acid.

It is also evident from Table III that there is a slight difference in activity between the unfractionated skin CSA and the purified CSA-B, the activity of the latter being somewhat lower. A determination of the radioactivity of the cetyl pyridinium-soluble fraction showed slightly higher specific activity. However, it is difficult to draw conclusions from such a small difference.

TABLE III

*Incorporation of radioactivity into mucopolysaccharides of skin after administration of glucose-6-C^{14}**

Material	Hyaluronic acid	"CSA fraction"	CSA-B
	*c.p.m.**	*c.p.m.**	*c.p.m.**
Entire molecule	52	32	29
Hexosamine	49		29
C-6 of uronic acid	292		174
2-Methylbenzimidazole derivative	5		3

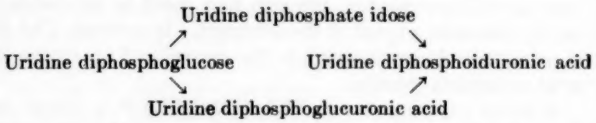
* Expressed as c.p.m. per mg. of $BaCO_3$.

DISCUSSION

Studies *in vivo* on the formation of urinary glucuronides have shown conclusively that glucose is converted to glucuronide without fragmentation of the carbon chain (11–13). Enzymatic studies of this conversion have revealed that the reaction proceeds in two stages. The first of these involves the formation of an "active glucuronyl" donor, uridine diphosphate glucuronic acid, from uridine diphosphate glucose (14); the second consists of the transfer of the glucuronyl group to an appropriate acceptor.

The glucuronic acid of streptococcal hyaluronic acid has been shown to incorporate radioactivity from glucose-6-C^{14} predominantly into the carboxyl group (8), which makes a direct conversion of the glucose carbon chain most likely. Markovitz *et al.* (27) have recently shown that tritiated uridine diphosphoglucuronic acid is incorporated into hyaluronic acid by an enzyme system derived from a strain of group A streptococci. The present work provides evidence that the glucuronic acid of skin hyaluronic acid also derives from unfragmented glucose.

The results of the present study clearly show that C^{14} activity from glucose-6-C^{14} is incorporated mainly into the carboxyl group of the L-iduronic acid. A direct transformation of the intact glucose carbon chain seems most likely. At present there are no data from known metabolic pathways to indicate how the inversion of configuration at C-5 takes place. The studies on the uridine diphosphogalactose-uridine diphosphoglucose interconversion and the formation of uridine diphosphoglucuronic acid suggest the possibility of a similar mechanism for the formation of L-iduronic acid. Whether inversion occurs before oxidation of C-6 is not known. There are no data which permit a choice between the two pathways indicated in the diagram below.



The C-4 epimerization in the glucose-galactose interconversion is considered to occur by way of an oxidation-reduction mechanism (28–30). In the glucose-idose transformation the C-5 epimerization is, however, complicated by the fact that C-5 lacks a hydroxyl group. Assuming that an oxidation-reduction mechanism is operative in this case, the possibility of a ring breakage or the formation of a Δ 4:5 enol intermediate must be considered. A dehydration between C-4 and C-5 followed by a rehydration with an accompanying change in configuration is also possible.

SUMMARY

A method for the isolation of highly purified chondroitinsulfuric acid-B from rat skin has been described. This involves exhaustive digestion of the chondroitinsulfuric acid mixture from skin with testicular hyaluronidase and precipitation of the

chondroitinsulfuric acid-B with excess cetyl pyridinium chloride. Under these conditions the digestion products remain in solution.

Hyaluronic acid and chondroitinsulfuric acid-B were isolated from rat skin after administration of glucose-6-C^{14}, and then the distribution of the radioactivity was studied. In both hyaluronic acid and chondroitinsulfuric acid-B the radioactivity was equally distributed between the hexosamine moiety and the C-6 of the uronic acid. The glucosamine moiety and the C-6 of glucuronic acid in hyaluronic acid each contained 40 per cent of the total activity. 5 per cent was present in the acetyl group. In chondroitinsulfuric acid-B 43 per cent of the radioactivity was found in the galactosamine moiety and 43 per cent in the C-6 of the L-iduronic acid moiety. 5 per cent was found in the acetyl group.

It is concluded that the L-iduronic acid of chondroitinsulfuric acid-B arises from glucose without splitting of the carbon chain.

Acknowledgment—The authors are grateful to Dr. Sara Schiller for her help in many aspects of this investigation.

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Effect of Protein Added *in Vitro* upon Insulin Degradation and Glucose Uptake by Muscle*

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(Received for publication, July 7, 1958)

Glucose tolerance is characteristically decreased in acromegaly or after the repeated administration of anterior pituitary growth hormone, but there are many reports that this hormone may actually stimulate glucose utilization by tissues in certain acute instances or in experiments *in vitro*. Thus, Park *et al.* (1) have observed a stimulation of glucose uptake by diaphragms removed from hypophysectomized rats shortly after the parenteral administration of growth hormone. Ottaway has shown that growth hormone added *in vitro* can stimulate the glucose uptake of diaphragms from normal rats (2), but not from rats with alloxan diabetes, unless these animals have first received an injection of insulin (3). Hence, the presence of insulin appears to be necessary for a demonstration of the insulinlike action of growth hormone.

Skeletal muscle can inactivate insulin (4) and degrade insulin-I^{131} (5). In the case of glucagon, the biological activity, as measured with liver slices *in vitro*, is augmented by the addition of insulin and other proteins (6, 7), and this may be explained by competition of these other proteins for a hepatic enzyme system which can inactivate glucagon (8, 9) and cleave glucagon-I^{131} (10).

These observations suggested that isolated diaphragm could inactivate small amounts of endogenous or exogenous insulin, and that growth hormone might enhance glucose utilization by sparing insulin from degradation. Experiments reported in the present work lend support to this hypothesis, and indicate that the enhancement of insulin action may not be a specific physiological action of growth hormone, but rather a property that is shared by other proteins and protein derivatives.

Since Randle and Whitney (11) have shown that growth hormone stimulates glucose uptake by isolated rat diaphragm more effectively in phosphate buffer than in bicarbonate buffer, phosphate buffer has been used in most of the present work. In addition, as it has been found previously that insulin-I^{131} may undergo adsorption to glassware, and that the extent of this adsorption is influenced by the presence of other proteins (12, 13), this phenomenon was studied again and measures were adopted to minimize this variable in the experiments on insulin degradation.

EXPERIMENTAL

Materials—A mixture of radioiodinated and nonlabeled crystalline zinc insulin was used as substrate in the assays of insulin degradation. Oxidized insulin was prepared from amorphous insulin of low zinc content by the method of Sanger and Thompson (14). Crystalline preparations of BPA[1] and glucagon were also used, as were highly purified α-casein, beef anterior pituitary growth hormone, Oxycel[2] purified porcine ACTH, and porcine growth hormone.[3]

Extract of Rat Diaphragm Acetone Powder—A 20 per cent tissue homogenate in 1.15 per cent KCl, pH 7.4, was prepared from the pooled diaphragms of male Sprague-Dawley rats weighing 250 to 350 gm., and the supernatant fluid obtained by centrifugation for 20 minutes at 9000 $\times$ *g* was precipitated with cold acetone, washed five times with acetone, and dried in a vacuum. The acetone powder was extracted with 1 ml. of 0.1 M Tris buffer, pH 7.4, per 50 mg. of powder for 1 hour in the cold room, and then centrifuged to remove insoluble material. Such extracts contained approximately 27 mg. of protein per ml. when analyzed by the biuret method of Robinson and Hogden (15). Dialysis of the extract, when performed, was against 100 volumes of 0.1 M Tris buffer for 4 hours, with one change of the bath fluid.

Adsorption of Insulin-I^{131} to Glass—0.2 μg. (3×10^{-5} μmole)[4] of insulin-I^{131} was incubated for 60 minutes in 12-ml. centrifuge tubes at 37° in 2 ml. of 0.05 M Tris buffer, pH 7.4. For growth hormone the pH was increased to 9 in order to dissolve the material, and a control solution of insulin-I^{131} alone was also run at this pH. After incubation the tubes were emptied and rinsed with H_2O, and the radioactivity remaining on the walls of the tubes and that which was in the combined fluid contents of each tube was counted as previously described (16). The degree to which the adsorption of insulin-I^{131} on the glass tubes was altered by the addition of more protein was calculated and expressed as a percentage of inhibition of adsorption.

Leaching of Diaphragms—Two types of buffered medium were

* This research was supported by Research Grants A-575 and A-761 of the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service.

[1] The abbreviations used are: BPA, bovine plasma albumin; ACTH, adrenocorticotropic hormone; Tris, tris(hydroxymethyl)-aminomethane buffer.

[2] Obtained from Parke, Davis and Company.

[3] Crystalline zinc insulin, amorphous insulin, and glucagon were gifts from Dr. O. K. Behrens of Eli Lilly and Company. Crystalline zinc insulin labeled with I^{131} was purchased from the Abbott Laboratories. Gifts of porcine ACTH and growth hormone were obtained from Dr. M. S. Raben, bovine growth hormone from Dr. C. H. Li, and α-casein from Dr. T. L. McMeekin. BPA was obtained from the Armour Laboratories.

[4] Calculations of molar concentrations were based upon the following values for molecular weights: α-casein, 100,000; BPA, 70,000; growth hormone, 46,000; insulin, 6,000; ACTH, 4,500; and glucagon, 3,500.

used. The bicarbonate medium contained the mixture of salts recommended by Gey and Gey (17); this was gassed with 5 per cent CO_2-95 per cent O_2 before use. The other medium was a modification of that described by Stadie *et al.* (18), and had the following composition: 0.04 M sodium phosphate, pH 7.4, 0.087 M NaCl, and 0.005 M $MgCl_2$. This solution was gassed with 100 per cent O_2 before incubation.

Hemidiaphragms from male or female Sprague-Dawley rats weighing 120 to 150 gm. were placed in cold buffer for 10 minutes after excision, and then blotted and transferred to Warburg vessels containing 1 ml. of fresh buffer to which had been added 1 mg. of BPA. For certain experiments, the hemidiaphragms were cut into thirds to produce sixths of diaphragms just before leaching. In any given experiment, procedures such as cutting of the tissue or alterations of the buffer medium were performed on one hemidiaphragm from each rat, and the contralateral hemidiaphragm was used as a control. Tissue from two rats, equivalent to two hemidiaphragms, was placed in each vessel. Incubations were carried out at 37° and were shaken 90 times per minute. After incubation, the media from similarly treated vessels were pooled, and chilled on ice. When dialysis was done, this was carried out against 100 volumes of the phosphate buffer for 4 hours, with one change of bath fluid, and the medium was then diluted to a convenient volume.

Degradation of Insulin-I^{131} by Acetone Powder Extract—The insulin substrate for these assays consisted of a trace quantity (0.1 to 0.3 μg.) of insulin-I^{131} plus a suitable amount of crystalline insulin; the total concentration of this mixture is stated in each experiment. 1 ml. of insulin substrate was incubated with varying amounts of diaphragm acetone powder extract in 2 ml. of 0.1 M Tris buffer, pH 7.4. The reaction was terminated by the addition of 2 ml. of 20 per cent trichloroacetic acid preceded by 1 ml. of a 2 per cent solution of lyophilized human plasma to act as additional protein carrier. The trichloroacetic acid-soluble and -insoluble materials were separated and counted as described earlier (16).

Effect of Diaphragm and of Diaphragm Incubation Medium upon Insulin-I^{131}—The direct effect of muscle segments upon insulin-I^{131} was studied by incubating these together in Warburg flasks in 2 ml. of phosphate buffered medium under O_2. After incubation the flasks were emptied and rinsed with 1 ml. of 2 per cent plasma and with water, and the pooled liquid from each flask was treated with trichloroacetic acid and counted as in the experiments described earlier.

When material that had been leached from diaphragms into phosphate buffered medium was being studied, this was incubated with insulin-I^{131} in open test tubes as in the tests with acetone powder extracts. However, when bicarbonate buffered media were included in the experiment, incubations were conducted in Warburg flasks after gassing the phosphate buffer with O_2 and the bicarbonate buffer with 5 per cent CO_2-95 per cent O_2. After incubation, the flasks were rinsed with 1 ml. of 2 per cent plasma and with water into centrifuge tubes. Trichloroacetic acid was then added to a final concentration of 5 per cent, and the mixture was processed and the radioactivity counted in the usual manner.

Biological Activity of Insulin—The action of insulin upon the isolated hemidiaphragms of male and female Sprague-Dawley rats weighing 120 to 135 gm. was studied by the method of Vallance-Owen and Hurlock (19) except that phosphate buffered medium was used in most instances instead of a bicarbonate medium. 2 ml. of medium containing 300 mg. of glucose per 100 ml. were added to each flask. One hemidiaphragm from each rat was incubated in the presence of insulin, whereas the contralateral hemidiaphragm was always used to measure the basal glucose uptake in the absence of added insulin. Other proteins, when present, were added to both the insulin-containing medium and the corresponding basal medium. In experiments in which bovine growth hormone was used, the medium in all flasks was adjusted to a pH of 7.6. The difference between the glucose uptake in the medium containing insulin and the corresponding basal medium was called the insulin effect. On any given day, a unit experiment was run in which the insulin effect in the presence of an added protein was compared with the insulin effect in the absence of that protein.

RESULTS

Adsorption of Insulin-I^{131} to Glassware—The data in Table I reveal that most of the proteins studied caused a marked diminution in the amount of insulin-I^{131} which was adsorbed to glass containers during incubation, but ACTH was notably less efficient than the other compounds in this regard. In the absence of carrier proteins, the adsorption of insulin-I^{131} varied and ranged from approximately 18 per cent to 30 per cent of the amount initially present in solution.

Degradation of Insulin-I^{131} by Muscle Acetone Powder Extract—The extract prepared from an acetone powder of pooled rat diaphragms was found to degrade insulin-I^{131}, and the rate of this degradation was approximately proportional to the amount of extract used, when substrate was present in sufficient excess

TABLE I

Effect of addition of proteins upon adsorption of insulin-I^{131} to glass

Each tube contained 3×10^{-5} μmole of insulin-I^{131}.

Protein added	Amount added	Inhibition of adsorption of insulin-I^{131}
	μmole	%
α-Casein	0.01	96
Pork growth hormone	0.01	93
BPA	0.01	91
Crystalline insulin	0.01	90
Glucagon	0.01	86
ACTH	0.01	45
ACTH	0.05	65

TABLE II

Effect of pH upon degradation of insulin-I^{131} by extract of rat diaphragm acetone powder

3 μg. of labeled insulin substrate were incubated with 0.05 ml. of an extract of acetone powder of rat diaphragm and 1 mg. of BPA in 2 ml. of 0.025 M potassium phosphate solution for 20 minutes, in duplicate, at 37°.

pH	Radioactivity in trichloroacetic acid supernatant
	%
6.0	7.9
7.0	21.0
7.5	22.1
8.0	17.7

TABLE III

Effect of dialysis and heating upon activity of rat diaphragm acetone powder extract

Extracts were prepared as described under "Experimental." One portion of undialyzed extract was heated in a boiling water bath for 10 minutes. 0.1 ml. of extract plus 30 μg. of labeled insulin substrate were incubated in 2 ml. of 0.1 M Tris, in quadruplicate, for 40 minutes.

Preparation	Radioactivity in trichloroacetic acid supernatant
	%
Undialyzed extract	14.2 ± 0.5*
Dialyzed extract	11.5 ± 0.3
Heated extract	0.36 ± 0.05

* Standard error of the mean.

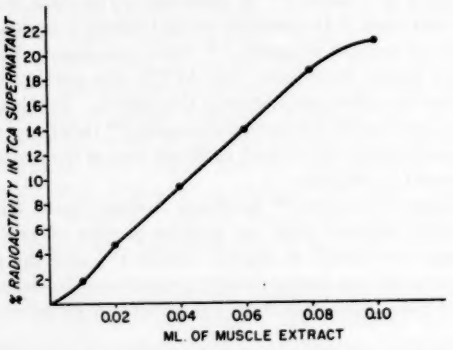

FIG. 1. Effect of concentration of diaphragm acetone powder extract upon the degradation of insulin-I^{131}. Assay of insulin-I^{131} degradation was conducted as described in the text. 1.5 μg. of labeled insulin substrate was incubated for 10 minutes with an extract of rat diaphragm acetone powder in 1 ml. of 0.05 M Tris, pH 7.4, with 1 mg. of BPA, in duplicate.

(Fig. 1). Degradation of insulin-I^{131} by the muscle extract proceeded optimally at approximately pH 7.5 in phosphate buffer (Table II). Most of the insulin-degrading activity of the extract survived dialysis for 4 hours, but activity was destroyed by heating in a 100° bath for 10 minutes (Table III). Thus, it appears that muscle homogenate which has been precipitated and washed with acetone contains little of the heat-stable insulin-degrading factor which has been described in muscle (20) and in liver (21, 13). Electrophoretic analysis of the trichloroacetic acid-soluble radioactive material resulting from the action of diaphragm powder extract upon insulin-I^{131} disclosed that this was not free iodide (Fig. 2). As the I^{131} which becomes soluble in trichloroacetic acid during incubation of the labeled substrate with diaphragm extract is presumably still attached to fragments of the insulin molecule, changes in the amount of trichloroacetic acid-soluble radioactivity may be used as a measure of the cleavage of insulin by the nondialyzable, heat-labile degradative system of such extracts.

Effect of BPA upon Degradation of Insulin-I^{131} by Extract of Rat Diaphragm Acetone Powder—When BPA was added to the reaction mixture, the rate of degradation of insulin-I^{131} by diaphragm extract was not affected if the concentrations of substrate and extract were adequately high (Table (IV). Similar results have been reported with rat liver extracts (13). BPA did not decrease the rate of degradation of insulin-I^{131} by diaphragm extract under any of the conditions tested. Indeed, when a dilute preparation of diaphragm extract was employed, the addition of BPA actually enhanced the degradation of insulin-I^{131} (Table IV), perhaps in part by retarding the denaturation of an insulin-degrading enzyme system. Hence, it was felt

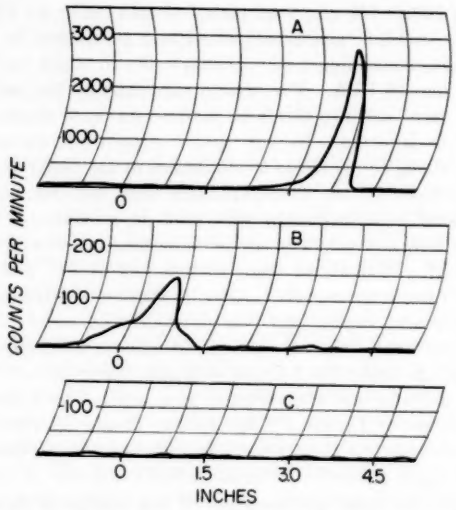

FIG. 2. Electrophoretic study of trichloroacetic acid-soluble material resulting from incubation of insulin-I^{131} with an extract of rat diaphragm acetone powder. Three different mixtures were incubated in duplicate for 30 minutes in 0.5 ml. of 0.025 M potassium phosphate, pH 7.4: *A*, 10 μc. of carrier-free NaI^{131} and 0.2 ml. of diaphragm extract; *B*, 24 μc. (2 μg.) of labeled insulin substrate and 0.2 ml. of diaphragm extract; *C*, 24 μc. of labeled insulin substrate alone. Each incubation tube also contained 0.5 mg. of BPA. After incubation the tubes were chilled on ice, and 0.1 ml. of a 10 per cent solution of KI, 0.3 ml. of 2 per cent plasma, and 0.3 ml. of 20 per cent trichloroacetic acid were added in rapid succession, with stirring. 10 mμl. of the supernatant fluid obtained by centrifugation were applied at the origin, *O*, of a strip of Whatman No. 1 filter paper, 1.5 inches in width, which had been moistened with 0.1 M potassium phosphate buffer, pH 7.5. Electrophoresis was carried out in the cold room for 90 minutes in horizontal boxes described by Grassmann and Hannig (22) at a potential of 200 volts, and radioactivity was counted on the dried strips with an end-window Geiger-Müller counter arranged for automatic scanning.

TABLE IV

Effect of BPA upon degradation of insulin-I^{131} by acetone powder extract of diaphragm

Incubation was carried out in 2 ml. of 0.1 M Tris, in duplicate.

Labeled insulin substrate	Diaphragm extract	BPA	Incubation	Radioactivity in trichloroacetic acid supernatant
μg.	*ml.*	*mg.*	*min.*	%
20	0.3	0	10	15.2
20	0.3	1	10	15.0
20	0.3	2	10	15.0
5	0.025	0	30	3.2
5	0.025	1	30	7.0

that BPA could be used to minimize losses of substrate and enzymes from dilute solutions during incubation and to decrease experimental variations caused by the effect of other proteins upon such losses.

Effect of Other Proteins upon Degradation of Insulin-I^{131} by Diaphragm Acetone Powder Extract—When various proteins other than BPA were incubated with insulin-I^{131} in the presence of an extract of rat diaphragm under conditions where the amount of extract limited the rate of reaction, it was found that the proteins could decrease the rate of degradation of the insulin-I^{131} (Table V). The pork growth hormone preparation was not very soluble at pH 7.4, so it was tested at a higher pH. Growth hormone exerted a small but definite effect in depressing the rate of degradation of insulin-I^{131}, and pork and beef growth hormone manifested similar degrees of activity.

Degradation of Insulin-I^{131} by Isolated Rat Diaphragm—The incubation of insulin-I^{131} with rat hemidiaphragms in a phosphate buffered medium resulted in an increase in trichloroacetic acid-soluble radioactive material in the medium, and the rate of this reaction was accelerated by cutting the diaphragms into smaller segments (Table VI).

Insulin does not accumulate rapidly within diaphragm or skeletal muscle after intravenous administration in rats (23), and even in the case of rat liver the ability to inactivate insulin is enhanced by homogenization and dilution of the tissue (24). If insulin-degrading factors could escape from rat diaphragms during incubation, they might more readily degrade insulin which is present in the surrounding medium. Therefore, it was of interest to see whether or not such a process of leaching could be demonstrated.

When rat hemidiaphragms were incubated in a buffered medium and then removed, it was found that the medium itself was, indeed, able to degrade insulin-I^{131}, and more insulin-degrading activity appeared when phosphate buffer was used than when the medium was buffered with bicarbonate (Table VII). BPA was added to the medium in order to help maintain in solution any small amounts of enzyme which might enter the medium from the diaphragms. A control study revealed that the insulin-degrading activity of an extract of diaphragm acetone powder was the same whether the assay was conducted in phosphate or bicarbonate buffered medium. As more of the insulin-degrading activity of diaphragm could be extracted with the phosphate medium, this solution was used for most of the studies on the leaching phenomenon.

More insulin-degrading activity appeared in the leaching medium when hemidiaphragms were cut into smaller portions before extraction (Table VIII); this might help to explain the greater ability of segmented diaphragms to degrade insulin-I^{131} (Table VI). The insulin-degrading activity which escaped from diaphragms into the medium persisted to some extent even after dialysis for 4 hours (Table IX), but heating of the dialyzed medium for 10 minutes destroyed this residual activity com-

TABLE V

Effect of proteins upon degradation of insulin-I^{131} by acetone powder extract of diaphragm

Data from 3 experiments, A, B, and C, are given. Each incubation vessel contained 2.5×10^{-4} μmole (1.5 μg.) of labeled insulin substrate, 1 mg. of BPA, and 0.05 ml. of acetone powder extract in 2 ml. of 0.1 M Tris. Incubation was for 10 minutes, in triplicate.

Protein added	Amount	pH of medium	Radioactivity in trichloroacetic acid supernatant		
			Experiment A	Experiment B	Experiment C
	μmole		%	%	%
None		7.4	14.7	16.3	
Glucagon	0.01	7.4	9.7	11.5	
α-Casein	0.01	7.4	4.0	3.6	
ACTH	0.01	7.4	1.0	1.2	
Oxidized insulin	0.01	7.4	1.0	1.1	
Crystalline insulin	0.01	7.4	0.9	0.9	
None		7.9	13.6		
Pork growth hormone	0.01	7.9	11.7		
None		9.0			12.0
Pork growth hormone	0.01	9.0			10.5
Pork growth hormone	0.02	9.0			9.0
Pork growth hormone	0.03	9.0			8.1
Beef growth hormone	0.02	9.0			9.4

TABLE VI

Degradation of insulin-I^{131} by isolated rat diaphragm

Each Warburg flask contained one hemidiaphragm or three sixths of diaphragms plus 4 μg. of labeled insulin substrate and 2 mg. of BPA in 2 ml. of phosphate buffered medium. Incubation was for 60 minutes, in quadruplicate.

Tissue	Mean dry weight of tissue per flask	Radioactivity in trichloroacetic acid supernatant
	mg.	%
Hemidiaphragms	29.2 ± 0.6*	37.7 ± 2.1*
Sixths of diaphragms	26.1 ± 1.2	46.9 ± 1.6

* Standard error of the mean.

TABLE VII

Leakage of insulin-degrading system from hemidiaphragms into phosphate and bicarbonate media

Leaching—In each experiment hemidiaphragms from six rats were extracted for 30 minutes in phosphate buffered medium in Warburg flasks, as described under "Experimental," and the contralateral hemidiaphragms were extracted with bicarbonate buffer. The separately pooled phosphate and bicarbonate media were then diluted with 0.5 volume of the corresponding buffer.

Degradation—The general procedure is described under "Experimental." 1 ml. of pooled diluted medium was incubated with 1 ml. of the same buffer containing 16 μg. (approximately 0.4 unit) of labeled insulin substrate and 1 mg. of BPA. Incubation for each experiment was in Warburg vessels for 60 minutes, in quadruplicate.

Experiment	Buffer medium	Total dry weight of diaphragms	Radioactivity in trichloroacetic acid supernatant
		mg.	%
A	Phosphate	135.8	11.7 ± 0.4*
	Bicarbonate	140.5	5.4 ± 0.1
B	Phosphate	176.9	11.5 ± 0.7
	Bicarbonate	188.0	6.3 ± 0.1

* Standard error of the mean.

TABLE VIII

Effect of segmentation of diaphragms upon leaching of insulin-degrading system

Leaching—Hemidiaphragms or sixths of diaphragms from 10 rats were extracted with phosphate buffered medium in Warburg cups for 30 minutes, after gassing with O_2, as described under "Experimental." The media were then pooled in two groups according to the type of tissue used.

Degradation—1 ml. of pooled medium was incubated in an open test tube with 1 ml. of phosphate buffer containing 2 μg. of labeled insulin substrate and 1 mg. of BPA, for 30 minutes, in quadruplicate.

State of diaphragms during extraction	Total dry weight of diaphragms	Radioactivity in trichloroacetic acid supernatant
	mg.	%
Hemidiaphragms	224.9	34.7 ± 0.4*
Sixth diaphragms	213.7	48.7 ± 0.6

* Standard error of the mean.

TABLE IX

Effect of dialysis and heating upon activity of insulin-degrading system leached from hemidiaphragms

Leaching—In Experiment A, hemidiaphragms from eight rats were extracted for 90 minutes in Warburg flasks with phosphate buffer under O_2, as described in "Experimental." Media were next pooled and an aliquot dialyzed for 4 hours. Finally, the media were diluted with 0.5 volume of phosphate buffer, and an aliquot of dialyzed medium was heated in a boiling water bath for 10 minutes. In Experiment B, hemidiaphragms from four rats were extracted for 90 minutes in phosphate medium containing 300 mg. of glucose per 100 ml. After dialysis against phosphate medium containing no glucose, the pooled media were diluted with 0.5 volume of phosphate buffer.

Degradation—In both experiments, 1 ml. of medium was incubated with 1 ml. of phosphate buffer containing 2 μg. of labeled insulin substrate and 1 mg. of BPA, aerobically, for 40 minutes, in triplicate.

Experiment	Medium	Radioactivity in trichloroacetic acid supernatant
		%
A	Undialyzed	40.1
	Dialyzed	12.2
	Dialyzed and heated	0.2
B	Glucose, dialyzed	13.3

pletely. The presence of glucose in the incubation medium did not prevent the efflux of the insulin-degrading system from diaphragms (Table IX). When α-casein, ACTH, or nonlabeled insulin were added to dialyzed incubation medium containing the leached insulin-degrading system, it was found (Table X) that the rate of degradation of insulin-I^{131} could be depressed in a manner similar to that found with extracts of acetone powder of diaphragm (Table V). The extent to which the destruction of insulin and the inhibition of this destruction by other proteins could also occur within muscle cells was not investigated.

Influence of Various Proteins upon Glucose Uptake by Isolated Rat Diaphragm—Insulin in a concentration of 10^{-4} unit per ml. exerted a definite effect upon the glucose uptake of rat

TABLE X

Effect of added proteins upon activity of dialyzed insulin-degrading system leached from hemidiaphragms

Leaching—Following the standard procedure, hemidiaphragms from 12 rats were extracted with phosphate buffer under O_2. The pooled media were then dialyzed and diluted with 0.1 volume of phosphate buffer.

Degradation—0.5 ml. of dialyzed medium was incubated in open test tubes with 1.5 μg. (2.5×10^{-4} μmole) of labeled insulin substrate and 1 mg. of BPA with or without the addition of 1×10^{-4} μmole of each of the carrier proteins listed in the table, for 90 minutes, in quadruplicate.

Protein added	Radioactivity in trichloroacetic acid supernatant
	%
None	36.6 ± 0.4*
α-Casein	14.8 ± 0.2
ACTH	3.20 ± 0.07
Insulin	2.10 ± 0.01

* Standard error of the mean.

TABLE XI

Effect of various proteins upon glucose uptake by isolated rat diaphragm

The procedure is described under "Experimental." All incubations were carried out in quadruplicate, and, except for Experiment A, phosphate buffer was used. The effect of adding insulin in a concentration of 10^{-4} unit per ml. (1.2×10^{-6} μmole per vessel) was tested in the absence or presence of the listed proteins which were added in a concentration of 10^{-2} μmole per vessel.

Experiment	Added protein	Insulin effect*	p-value†
A	None; phosphate buffer	1.8 ± 0.6‡	
	None; bicarbonate buffer	4.8 ± 0.8	$p < 0.05$
B	None	3.1 ± 0.6	
	BPA	3.6 ± 0.3	$0.2 < p < 0.5$
C	None	2.5 ± 0.3	
	BPA	1.9 ± 0.2	$0.2 < p < 0.5$
D	None	1.8 ± 1.0	
	ACTH	6.5 ± 1.1	$p < 0.02$
E	None	1.2 ± 0.7	
	Oxidized insulin	3.8 ± 0.2	$p < 0.02$
F	None	1.6 ± 0.2	
	α-Casein	4.3 ± 0.7	$p < 0.02$
G	None	1.5 ± 0.4	
	Glucagon	3.2 ± 0.5	$p < 0.05$
H	None	2.8 ± 0.5	
	Beef growth hormone	6.3 ± 1.2	$p < 0.05$

* The insulin effect was the difference in glucose uptake by diaphragm in the presence and absence of insulin, expressed as mg. of glucose per 100 ml. of medium per 10 mg. of dry weight of diaphragm, per 90 minutes of incubation.

† The statistical significance of the difference between the pair of means in each experiment is expressed as the *p*-value.

‡ Standard error of the mean.

diaphragm in bicarbonate buffer, but had little or no effect in phosphate buffer (Table XI). In preliminary experiments under a variety of conditions, the addition of ACTH, oxidized insulin, α-casein, glucagon or growth hormone did not alter the glucose uptake by rat hemidiaphragms when the tests were conducted in bicarbonate buffer, with or without the addition of insulin. It is possible that further search would have revealed conditions under which an effect could be demonstrated in bicarbonate buffer (25), but since positive results were readily obtained with phosphate buffered medium, this was used for the experiments reported here. It can be seen in Table XI that ACTH, oxidized insulin, α-casein, glucagon and growth hormone were capable of producing a significant increase in the glucose uptake of diaphragm from a phosphate buffered medium in the presence of 10^{-4} unit per ml. of insulin. The action of these proteins was, unfortunately, too limited and variable to permit an accurate gradation of their effectiveness, but the impression was gained that ACTH was more active than either growth hormone or glucagon in promoting glucose uptake. The control hemidiaphragms were not paired in such a way as to permit precise evaluation of the effect of adding proteins upon the basal glucose uptake of hemidiaphragms in the absence of insulin, but no appreciable effect of this type was noted in any of the experiments. BPA differed from these other proteins in that it did not cause a significant alteration of glucose uptake in the presence of insulin. BPA was not added routinely to all of the incubation flasks used in the bioassays, in order that the technique might be more comparable to that of earlier workers who did not use BPA to protect against adsorption of insulin to glassware.

DISCUSSION

The observations that growth hormone can stimulate glucose uptake when added *in vitro* to diaphragms from normal (2) or hypophysectomized (1) rats, and that an injection of growth hormone can produce hypoglycemia in acutely depancreatized dogs (26) or rats (1) suggest that these actions do not depend upon an increase in the rate of insulin secretion. However, there is evidence that a minimal amount of insulin must be present in the tissues in order for growth hormone to stimulate glucose utilization (3, 27). Other proteins have also been noted to exert insulinlike effects. Thus, Westermeyer and Raben (28) noted that ACTH transiently lowered the blood sugar of mice, and Randle (25) discovered that crystalline glucagon added *in vitro* stimulated glucose uptake by rat diaphragm and enhanced the action of added insulin.

The results of the present studies indicate that muscle can destroy insulin to a significant extent during incubation *in vitro*, and suggest that the insulinlike action of certain proteins may be related to a sparing of insulin from degradation. The observation that the degradation of insulin by muscle extracts was impaired by the addition of other proteins is indirect evidence that the process may involve proteolysis. In the case of a similar phenomenon observed with liver extracts, it has been suggested that certain proteins, or peptide derivatives of these, may retard the destruction of insulin by acting as competitive substrates for a proteolytic enzyme system (29, 30).

Zierler (31) has described the efflux of aldolase from the isolated skeletal muscle of rats during incubation *in vitro*, and in the present work it was shown that a nondialyzable, heat-labile system which degrades insulin could be leached from rat diaphragm. On the other hand, the partial loss of degradative activity during dialysis suggests that a dialyzable substance such as glutathione may also have contributed to the insulin-degrading activity of the effluent (20, 32). As preliminary experiments indicated that the degradation of insulin-I^{131} by glutathione was not appreciably affected by the addition of other proteins, attention was focused upon the enzymatic destruction of insulin, which was markedly influenced by certain proteins.

Although ACTH was less effective than BPA in preventing adsorption of insulin-I^{131} to glassware, it was more potent in depressing the degradation of insulin-I^{131} and in enhancing the glucose uptake by diaphragm. This suggests that, under the condition of these experiments, degradation of insulin exerted a greater influence upon the biological activity of insulin than did adsorption to glass. However, others have reported that BPA can stimulate glucose uptake by rat diaphragm (33). Since BPA may protect enzymes from denaturation in dilute solutions, it would not be surprising if, under appropriate circumstances, it should even accelerate the inactivation of insulin by isolated muscle.

In the light of these observations it does not seem necessary to postulate further that proteins such as ACTH, growth hormone or α-casein stimulate the glucose uptake of muscle by a direct action upon carbohydrate metabolism, or by increasing the sensitivity of the tissue to insulin, but these possibilities have not been ruled out for each substance.

Whereas one might agree that a sparing of insulin from destruction should augment its biological activity, several conditions must be satisfied *in vitro* before such an effect can actually manifest itself. For instance, insulin in a concentration of 10^{-4} or 10^{-5} unit per ml. produces a barely measurable effect upon the glucose uptake of rat diaphragm; unless this minimal amount of insulin is being broken down *in vitro*, an inhibition of the degradation may not augment the glucose uptake.

If enough insulin is present, the rate of degradation will be a function of the activity of the degrading system, which in turn may be affected by factors such as the nature of the buffer medium used, and the amount of cutting or trauma to which the tissues have been exposed. Furthermore, changes in the sensitivity of the diaphragms occasioned by procedures such as hypophysectomy or special handling of the muscle may govern the amount of insulin which has to be spared in order to be detectable. Again, at any given concentration of insulin, the nature of the bioassay is such that it cannot readily detect less than a 2-fold change in level. Therefore, at least half of the insulin present has to be inactivated by the muscle before an inhibition of degradation can play an appreciable role. When a very large amount of insulin is added to the medium, the degradative system of muscle may not be capable of attacking as much as 50 per cent of this during the incubation; then, sparing of this fraction would not influence the glucose uptake. Not only must the conditions permit a certain degree of inactivation of insulin, but the growth hormone or other protein agent which is used must be added in high enough concentration to produce an adequate depression of degradation. These stipulations may help to explain some of the situations in which various proteins have or have not been able to increase the utilization of glucose by isolated tissues.

The relative importance of intracellular and extracellular sites of insulin catabolism upon the bioassay of insulin with isolated

rat diaphragm remains unknown. However, it has been shown that the degradative system which escapes in a 30-minute period into phosphate medium can by itself degrade approximately 4×10^{-2} unit of insulin during subsequent incubation for 60 minutes. In the bioassay experiments, only 2×10^{-4} unit of insulin was added to each vessel. Hence, the medium alone should have been able to destroy most of this small amount within the 90 minutes allowed for incubation. As far as the efficiency of the inhibitors in concerned, in the studies with acetone powder extract, proteins such as ACTH and oxidized insulin caused marked inhibition of insulin-I^{131} degradation when the ratio of molar concentration of inhibitor to insulin was 40:1. In the bioassay experiments, the various proteins were added in approximately 8000-fold molar excess over insulin, and this should have been sufficient to impede affectively the degradation of insulin in the medium. Efforts were mainly directed towards obtaining a positive effect *in vitro*, and not towards reproducing physiological conditions.

One interesting finding was that insulin-degrading activity could be leached from diaphragms to a greater extent by phosphate buffer than by bicarbonate buffer. This may help to explain the greater biological activity of insulin in bicarbonate buffer, and might also be related to the observation that growth hormone increased the glucose utilization more readily in phosphate buffer than in bicarbonate buffer (3, 11).

The observation that insulin-I^{131} was broken down more rapidly by diaphragms that had been cut into smaller pieces is in accord with the reports (34, 35) that, when hemidiaphragms were segmented, the activity of exogenous insulin was diminished.

It is hoped that the results presented here will help to clarify some of the alterations of glucose metabolism which are associated with the addition of proteins such as growth hormone, ACTH, and glucagon to muscle *in vitro*.

SUMMARY

It has been shown that isolated rat diaphragm and extracts of diaphragm can degrade insulin-I^{131}, and the data suggest that enzymatic factors are important in this process. Corticotropin, oxidized insulin, α-casein, glucagon, and growth hormone were able to inhibit the degradation of insulin-I^{131} and to augment the utilization of glucose by rat diaphragm *in vitro*. Evidence is presented which supports the hypothesis that the effect on glucose utilization is related to a sparing of insulin from degradation.

Acknowledgments—The authors are grateful to Dr. John Vallance-Owen for helpful suggestions in the performance of the bioassays. They are indebted to Miss Ruth Miller and Mrs. Pearl Forth for valuable technical assistance.

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Metabolic Adaptations in Higher Animals

IV. EFFECT OF THE ETHIONINE:METHIONINE RATIO OF THE DIET ON GLUCOSE-6-PHOSPHATASE ADAPTATION*

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(Received for publication, July 11, 1958)

Ingestion of a diet containing 40 per cent or more of sucrose causes a rapid rise in liver glucose-6-phosphatase activity[1] in the rat (1). The high enzyme activity, which has been interpreted as an adaptive response, is maintained for several weeks at least, if the rat is kept on a diet high in sucrose (2). The glucose-6-phosphatase adaptation is thought to be a result of enzyme synthesis rather than activation of existing proenzyme (3).

The ingestion of ethionine will cause a loss of weight in the rat (4) and ethionine added to the medium will inhibit the growth of microorganisms (5); as these effects can be reversed by methionine supplementation, they have been attributed to an ethionine-methionine antagonism. Also, the formation of adaptive enzymes in bacteria (6) and in the rat (7) can be inhibited by ethionine; so it was thought that, if the glucose-6-phosphatase adaptation represented protein synthesis, it should be at least partially inhibited in rats fed a diet containing ethionine.

EXPERIMENTAL

Animals and Diets—Male Sprague-Dawley rats weighing 100 to 200 gm. were used in all experiments. All animals were fed *ad libitum* throughout the experiments. Rats were partially depleted of protein by fasting them for 2 days and then feeding them a diet containing 8 per cent of casein for 5 days before the start of the experiment. The nondepleted rats were fed a diet containing 25 per cent of casein for 3 days before they were given the experimental diets. The composition of the various diets used is shown in Table I.

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from the Research Committee of the Graduate School from funds provided by the Wisconsin Alumni Research Foundation; and from the National Institute of Arthritis and Metabolic Diseases, of the National Institutes of Health, United States Public Health Service. Some of the crystalline vitamins were kindly supplied by Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey.

[1] The following simplified expressions are used in the text and tables: liver glucose-6-phosphatase activity, glucose-6-phosphatase activity per gm. of liver; glucose-6-phosphatase activity per unit of body weight, total liver glucose-6-phosphatase activity times 100 divided by the body weight; specific activity, liver glucose-6-phosphatase activity per gm. of liver protein; relative liver weight, weight of the liver times 100 divided by the body weight.

Assay Procedure—In the experiments in which a single leve of inhibitor was used, groups of rats were killed after they had been fed the experimental diets for 1, 2, and 4 days; in the experiments in which various levels of inhibitor were used, the groups were killed after 1 and 2 days only. The livers were completely removed, weighed, and placed in ice. A portion of the chilled liver, weighed to the nearest milligram on a torsion balance, was homogenized at 0–4° in 40 volumes of 0.1 M potassium citrate buffer, pH 6.5. The homogenate was filtered through cheesecloth and the filtrate was used for the glucose-6-phosphatase assay. A portion of the homogenate was diluted for the protein determination (9). For the glucose-6-phosphatase assay 0.1 ml. of 0.06 M K_2 glucose-6-P, pH 6.5, was added to 0.1 ml. of the homogenate, both of which had been preincubated at 37.5° for 5 minutes. After incubation at 37.5° for 15 minutes, the reaction was stopped by adding 2 ml. of 10 per cent trichloroacetic acid. The activity was estimated by measuring the amount of inorganic phosphate released (10). These assay conditions produced zero order kinetics for values up to twice the greatest values reported.

RESULTS

Enzyme Unit—The amount of enzyme which causes the release of 1 μmole of inorganic phosphate from glucose-6-phosphate in 1 minute at 37.5° and pH 6.5 is defined as 1 unit of glucose-6-phosphatase. Values for glucose-6-phosphatase activity per gm. of liver, per unit of body weight, and per unit of liver protein are given either in absolute units or as a percentage of the control values. Standard errors are given for values calculated per unit of body weight, since a true metabolic adaptation of a system that provides a substrate for all body tissues should result in a change in the activity of the system on a body weight basis.

When protein-depleted rats were fed the Dextrin I diet containing 1 per cent of ethionine, the glucose-6-phosphatase activity per unit of body weight and the specific activity were unaltered (Table II). The relative liver size decreased but this was compensated by an increase in the liver glucose-6-phosphatase activity. However, when 1 per cent of ethionine was included in the Sucrose I diet, the glucose-6-phosphatase activity per unit of body weight and the specific activity were well below values for rats receiving the Sucrose I diet containing no ethionine. The liver glucose-6-phosphatase activity was only slightly lower but, as in the case of rats fed the dextrin diet containing ethionine, there was a marked decrease in relative

TABLE I

Composition of diets

Diet	I	II	III	IV	V	VI
Salts and vitamins (8)	5.0	5.0	5.0	5.0	5.0	5.0
Corn oil	5.0	5.0	5.0	5.0	5.0	5.0
Casein	8.0	4.0		9.0	18.0	27.0
Carbohydrate*	82.0	86.0	90.0	80.7	72.0	63.0
DL-Methionine				0.3		

* One series of diets contained sucrose, the other dextrin. The dextrin was prepared by heating moist cornstarch in an autoclave at 121° for 3 hours.

liver size. The values for glucose-6-phosphatase activity per unit of body weight for the group fed the Sucrose I diet containing 1 per cent of ethionine and for the groups fed the Dextrin I diet, with or without ethionine, were the same. This was also true of the values for specific activity of glucose-6-phosphatase for these three groups. In contrast, the values for rats receiving the Sucrose I diet containing no ethionine were higher. The total milligrams of liver protein per 100 gm. of body weight for the various groups were: Dextrin I, 520, Dextrin I + 1 per cent ethionine, 518, Sucrose I, 544, Sucrose I + 1 per cent ethionine, 555.

In the next experiment rats previously partially depleted of protein were fed a protein-free diet (Table III). The values for glucose-6-phosphatase specific activity, and the values for activity per unit of body weight, were about the same for the Dextrin III, Dextrin III + 1 per cent ethionine, and Sucrose III + 1 per cent ethionine groups. However, the increase in the glucose-6-phosphatase activity of rats fed the protein-free diet containing sucrose was much less than that observed in rats that were fed a similar diet containing 8 per cent of casein (Table II). The response to sucrose in rats fed the protein-free diet was significant only at the 5 per cent level, whereas, the response to sucrose when the diet contained 8 per cent of casein was significant at less than the 1 per cent level.

The effect of administering ethionine to rats that had not been depleted of protein was next investigated and the results are shown in Table IV. Ethionine prevented the adaptation to the sucrose diet in well fed animals just as it did in depleted animals. The values for glucose-6-phosphatase activity per unit of body weight for all groups in this experiment were higher than those obtained in the previous experiments, but the effect of ethionine was the same (Tables II and III). The values for liver protein were higher in this experiment and those for glucose-6-phosphatase specific activity were lower. In view of these results, the rats used in the remainder of the experiments were not depleted.

The effects on the glucose-6-phosphatase adaptation of feeding diets that contain 9 per cent of casein plus 0.3 per cent methionine, 18 per cent of casein, or 27 per cent of casein, to which various amounts of ethionine had been added, are shown in Table V. The glucose-6-phosphatase activity per unit of body weight decreased in every case as the amount of ethionine added to the diet was increased. Each decrease in glucose-6-phosphatase activity per unit of body weight was accompanied by a decrease in specific activity. The ratios of activity per unit of body weight to specific activity were between 1.00 to 1.25, 0.93 to 1.07, and 0.89 to 1.13 with average values of 1.13 ± 0.03, 1.00 ± 0.02, and 0.98 ± 0.04 for the 9 per cent, 18 per cent, and 27 per cent casein groups, respectively. This indicates that the total liver protein per unit of body weight remained fairly constant. The glucose-6-phosphatase adaptation was completely inhibited, both on the basis of glucose-6-phos-

TABLE II

Effect of ethionine on glucose-6-phosphatase adaptation in protein-depleted rats fed diet containing 8 per cent of casein

Diet	Glucose-6-phosphatase activity			Protein	Relative liver size
	units/100 gm. body weight	*units/ gm. liver*	*units/ gm. liver protein*	*mg./gm. liver*	
Dextrin I	68 ± 4*	15.9	131	121	4.3
Sucrose I	94 ± 7	19.2	173	111	4.9
Dextrin I + 1% ethionine	65 ± 4	17.7	127	140	3.7
Sucrose I + 1% ethionine	70 ± 3†	18.7	125	150	3.7

* Standard error of the mean; each value is the average of six animals.

† The difference in activity between the dextrin and sucrose groups caused by the addition of ethionine showed a probability of less than 0.01.

TABLE III

Effect of ethionine on glucose-6-phosphatase adaptation in protein-depleted rats fed protein-free diet

Diet	Glucose-6-phosphatase activity		Specific activity	Protein	Relative liver size
	units/100 gm. body weight	*units/ gm. liver*	*units/gm. liver protein*	*mg./gm. liver*	
Dextrin III	63 ± 4*	14.7	106	139	4.3
Sucrose III	73 ± 2	16.9	119	142	4.4
Dextrin III + 1% ethionine	65 ± 5	18.3	100	183	3.6
Sucrose III + 1% ethionine	66 ± 4	20.2	104	194	3.3

* Standard error of the mean; each value is the average of six animals.

TABLE IV

Effect of ethionine on glucose-6-phosphatase adaptation in non-depleted rats fed diet containing 4 per cent casein

Diet	Glucose-6-phosphatase activity		Specific activity	Protein	Relative liver size
	units/100 gm. body weight	*units/ gm. liver*	*units/gm. liver protein*	*mg./gm. liver*	
Dextrin II	80 ± 4*	15.9	84	190	5.1
Sucrose II	110 ± 5	19.3	103	188	5.7
Dextrin II + 1% ethionine	76 ± 6	20.0	81	248	3.7
Sucrose II + 1% ethonine	79 ± 3†	21.8	86	254	3.6

* Standard error of the mean; each value is the average of six animals.

† The difference in activity between the dextrin and sucrose groups caused by the addition of ethionine showed a probability of less than 0.01.

phatase activity per unit of body weight and specific activity, when 1.6 per cent of ethionine was included in the diets containing 9 per cent or 18 per cent of casein, and when 2.4 per cent of ethionine was included in the diet containing 27 per cent of casein. An increase in the ethionine level from 1.6 to 1.7 per cent in the diet containing 9 per cent of casein plus methionine did not cause a further reduction in glucose-6-phosphatase activity.

The weight change and food consumption of five animals receiving the Sucrose VI diet plus 2.4 per cent ethionine were measured for 2 days, the normal duration of the experimental period. The following are the 2-day results of this experiment: original weight 158 ± 11 gm.; weight change −26 ± 1 gm.; food consumption 13.4 ± 1.2 gm. The ingestion of this amount of food, containing over 60 per cent of sucrose, is more than sufficient to bring about the glucose-6-phosphatase adaptation (1).

Inasmuch as 9 gm. of casein contain about 0.3 gm. of methionine (11), the diet containing 9 per cent of casein plus 0.3 per cent of methionine, and that containing 18 per cent of casein, should both contain about 0.6 per cent of methionine. The 27 per cent casein diet would contain about 0.9 per cent of methionine. In order to inhibit the glucose-6-phosphatase adaptation completely, 1.6 per cent of ethionine was required in the diets containing 0.6 per cent of methionine and 2.4 per cent of ethionine in the diet containing 0.9 per cent of methionine. The ratio of ethionine to methionine required to cause complete inhibition was approximately 2.7 in each case.

The effect on the glucose-6-phosphatase activity of transferring animals fed the 27 per cent casein-sucrose diet (Sucrose VI) for 2 days, to either the Sucrose VI diet plus 2.4 per cent ethionine or the Dextrin VI diet, is shown in Table VI. Glucose-6-phosphatase activity per unit of body weight fell on the first day in both cases; there was also a drop in liver glucose-6-phosphatase activity and relative liver size. The glucose-6-phosphatase specific activity of the group receiving ethionine also fell below that of its sucrose control, but the values for the dextrin group and its sucrose control were not much different. By the second day, the values for glucose-6-phosphatase activity, and glucose-6-phosphatase specific activity for both of the experimental groups had fallen from the first day values, and were within the range obtained with animals fed dextrin diets for long periods of time (2, 3). Neither in this experiment nor in the experiments in which ethionine was used to inhibit the glucose-6-phosphatase adaptation did the values for glucose-6-phosphatase activity per unit of body weight fall below that for control animals.

TABLE V

Effect of various levels of ethionine on glucose-6-phosphatase adaptation of rats fed diets containing 9 per cent, 18 per cent, or 27 per cent of casein and high level of sucrose

Ethionine in diet	Glucose-6-phosphatase activity*					
	9% casein + 0.3% methionine		18% casein		27% casein	
%	*per 100 gm. body weight*	*per gm. liver protein*	*per 100 gm. body weight*	*per gm. liver protein*	*per 100 gm. body weight*	*per gm. liver protein*
0.00 (dextrin)	100 ± 3*	100	100 ± 8	100	100 ± 2	100
	(68 ± 2)	(79)	(73 ± 6)	(81)	(64 ± 1)	(74)
0.00	155 ± 3	137	170 ± 7	159	177 ± 12	156
0.15	156 ± 12	125				
0.25	147 ± 8	127	153 ± 2	159		
0.50	137 ± 6	112	140 ± 6	144		
1.00	124 ± 4	109	122 ± 8	119		
1.50	120 ± 7	93	122 ± 2	131		
1.60	95 ± 3	94	98 ± 6	96		
1.70	95 ± 7	93				
2.25					114 ± 8	117
2.40					104 ± 5	116
2.55					96 ± 5	108

* All values are reported as a percentage of the value for the group fed the dextrin diet. Each value represents the mean ± standard error for 4 rats. The numbers in parentheses are the average glucose-6-phosphatase values for the dextrin group in units, as defined in the text.

TABLE VI

Rate of loss of glucose-6-phosphatase activity gained during adaptation to high sucrose diet

Diet	Glucose-6-phosphatase activity			Protein	Relative liver size
	per 100 gm. of body weight	*per gm. of liver*	*per gm. of liver protein*	*mg./gm. liver*	
	1 day after dietary transfer				
Sucrose VI	101 ± 3*	17.2	102	168	5.9
Sucrose VI + 2.4% ethionine	82 ± 2	15.5	86	180	5.3
Sucrose VI	109 ± 7	16.3	90	182	6.7
Dextrin VI	84 ± 5	14.15	86	165	5.9
	2 days after dietary transfer				
Sucrose VI	99 ± 4	15.8	85	186	6.3
Sucrose VI + 2.4% ethionine	66 ± 3	14.45	73	198	4.6
Sucrose VI	111 ± 7	17.1	98	174	6.5
Dextrin VI	64 ± 3	12.3	75	165	5.2

* Standard error of the mean; each value is the average of five animals. All animals were fed the Sucrose VI diets for 2 days before the dietary transfers.

DISCUSSION

The inclusion of 1 per cent of ethionine in a diet high in sucrose completely inhibited the glucose-6-phosphatase adaptation; therefore, the increase in liver glucose-6-phosphatase activity in response to sucrose ingestion is evidently a true protein synthesis rather than an activation of either existing enzyme or an inactive precursor. Another point in favor of the adaptation representing protein synthesis is that, in contrast to the highly significant response to sucrose in groups fed a diet containing protein, those with a low protein reserve, receiving a protein-free diet containing sucrose, showed only a slight increase in glucose-6-phosphatase activity above the value for animals receiving a protein-free diet containing dextrin ($p = 0.05$) (Table III).

Also, the effect of ethionine ingestion was not the same as that of fasting. The values for glucose-6-phosphatase specific activity and activity per unit of body weight for groups receiving dextrin, dextrin plus ethionine, or sucrose plus ethionine were essentially the same. The rats that ingested ethionine must, therefore, have lost body weight and liver protein at about the same rate. During fasting, on the other hand,

glucose-6-phosphatase is conserved at the expense of the total liver protein.

The inhibition of the glucose-6-phosphatase adaptation by ethionine depends upon the ethionine-methionine ratio in the diet and is independent of the protein level, at least up to 27 per cent of casein. Each increase in glucose-6-phosphatase activity per unit of body weight, caused by a reduction in the ethionine-methionine ratio, was accompanied by a corresponding increase in specific activity indicating that under these circumstances glucose-6-phosphatase was synthesized preferentially to total liver protein. It is, of course, possible, since ethionine is known to be incorporated into rat liver protein (12), that a metabolically inactive protein, similar to glucose-6-phosphatase but containing ethionine, might be formed in rats receiving this antagonist. Such a protein would not be measurable as glucose-6-phosphatase.

The additional glucose-6-phosphatase which is formed rapidly in response to a high sucrose diet also disappears rapidly when the stimulus, such as sucrose, is removed; or continued synthesis of the active enzyme is prevented by ethionine. In both cases roughly half of the extra glucose-6-phosphatase activity had disappeared within 1 day, and the remainder by the second day. This indicates that there is continuous breakdown of the extra glucose-6-phosphatase; however, the ingestion of a high level of ethionine failed to decrease the glucose-6-phosphatase activity of rats fed a diet containing either sucrose or dextrin below normal values. These observations suggest that there may be two types of glucose-6-phosphatase. One type would seem to have a very slow turnover rate. This would account for the failure of 7 days of starvation (13) or 4 days of feeding high levels of ethionine in the diet to reduce the glucose-6-phosphatase activity below a certain minimum. The second type, which is synthesized in response to a glucogenic stress, would appear to be an enzyme with a very rapid turnover rate. This would account for its rapid disappearance when high levels of ethionine are included in the sucrose diet, or the stimulus causing the glucose-6-phosphatase response is removed. This hypothesis, which is consistent with the results presented and with those of other workers, can be tested by experiment.

SUMMARY

1. The inclusion of 1 per cent of ethionine in diets containing 8 per cent or less of casein as the sole source of protein completely inhibits the glucose-6-phosphatase adaptation to a high sucrose diet in rats.

2. The degree of inhibition of the glucose-6-phosphatase adaptation depends upon the ethionine to methionine ratio in the diet, with a ratio of 2.7 or above producing complete inhibition.

3. The increased glucose-6-phosphatase activity caused by the ingestion of a high sucrose diet disappears within 2 days when sufficient ethionine is added to the diet or dextrin is substituted for the sucrose.

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Coupling of Oxidation of Substrates to Reductive Biosyntheses*

II. LOCATION OF DEUTERIUM IN GLYCOGEN FORMED FROM DL-2-DEUTERIOLACTATE

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(Received for publication, July 11, 1958)

When lactate labeled with deuterium in the α position is administered to rats that have been previously fasted, the glycogen of the liver becomes enriched with deuterium, which indicates that the α-hydrogen atom of lactate is utilized in the reductive synthesis of carbohydrate (1). Although this observation is most readily interpreted as evidence of coupling of the oxidation of lactate to the reduction of phosphoglycerate, it would seem to be desirable to obtain more direct evidence that deuterium-labeled triose phosphate is indeed formed in the course of oxidation of α-deuteriolactate.

The present report deals with experiments bearing on this question. Degradation of the glucose obtained from the liver glycogen of animals which had received DL-α-deuteriolactate established the fact that the deuterium was located in the 4 and 6 positions. The presence of deuterium in the 4 position is in accord with the view that the α-hydrogen atom of lactate is a precursor of the hydrogen atom in the 1 position of Gl-3-P.[1] The observation that deuterium is present also in the 6 position provides evidence of the utilization of the α-hydrogen atom of lactate in another reductive step in the synthesis of carbohydrates. It is suggested that this step is the coupling of the oxidation of lactate to the reductive synthesis of malate.

EXPERIMENTAL

The glycogen used in the present studies was obtained from the livers of rats that had been previously fasted and had received α-deuteriolactate prepared and administered as described earlier (1). Table I presents the deuterium concentrations of the lactate, the yields of glycogen, and the deuterium concentrations of the glycogen and body water.

Preparation of Derivatives

Unless the data were available in the literature the degradative procedures of the present studies were carried out with the unlabeled compound in a medium containing D_2O in order to establish the extent of exchange between carbon-bound hydrogen of a particular product of degradation (or its derivative) and the hydrogen of the medium. The data of these studies showed that none of the procedures so tested results in significant labilization of carbon-bound hydrogen in the desired products.

Preparation of Glucose from Glycogen—Glucose was obtained by hydrolysis of the labeled glycogen in 1 M HCl at 100° for 3 hours. The reducing equivalents of the hydrolysates as measured by the Nelson-Somogyi method (2) corresponded to 98 and 92 per cent of the quantities of glucose expected from hydrolysis of the glycogen of Experiments L9 and L10, respectively. The hydrolysates were freed from the major proportion of HCl by repeated evaporations in a vacuum, and the resulting material was deionized by treatment with Amberlite IR-4B and IR-120(H). A small amount of residual color in the preparations was removed by charcoal. The yields of the derivatives and the ease with which crystalline products were obtained were substantially greater when the hydrolysates were completely deionized than when these steps were omitted.

Synthesis of α-Methyl Glucoside—The syrup obtained by evaporating a suitable aliquot of the labeled glucose solution was dried for several days in a vacuum desiccator containing P_2O_5. The dry, glassy material was then rapidly transferred with the aid of the appropriate quantity of 3 per cent HCl in methanol to a Liebhof urea tube in which subsequent procedures were conducted. The conditions which prevailed for methylation were those described by Boothroyd *et al.* (3). On completion of the reaction the solution was decolorized with charcoal and evaporated to a syrup in a vacuum at 40°. This was placed for 24 hours in a vacuum desiccator which contained soda lime. Crystallization of the glucoside was initiated by seeding. After recrystallization from 1:1 ethanol-methanol the product melted at 163° (uncorrected). When analyzed by the Nelson-Somogyi procedure the material was found to contain 0.6 per cent of unreacted glucose.

Oxidation of Glucose to Potassium Gluconate—For the purpose of oxidizing glucose to potassium gluconate an appropriate aliquot of the solution prepared as described above was evaporated to a syrup. Oxidation of this material by means of the procedure of Moore and Link (4) yielded potassium gluconate in amounts which compared favorably with those obtained when authentic crystalline glucose is used as starting material. The recrystallized product decomposed between 175–180° and was dried for 8 hours in a vacuum at 100° over P_2O_5 before combustion for deuterium analysis.

Periodate Oxidation of α-Methyl Glucoside—Formic acid with the hydrogen atom present in position 3 of glucose was obtained by oxidation of α-methyl glucoside with periodic acid according to the directions of Boothroyd *et al.* (3). After elution from Amberlite IR-4B the formic acid was steam-distilled and titrated with standard NaOH. From 1 mmole of glucoside was obtained 0.66 mmole of formic acid. The neutralized solution was then

* Aided by grants from the National Science Foundation and the Sugar Research Foundation.

[1] The abbreviations used are: Gl-3-P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; and fructose-DP, fructose-1,6-diphosphate.

TABLE I

Deuterium concentration of liver glycogen after administration of DL-*α-deuteriolactate*

Experiment No.	Deuterium concentration of lactate	Liver glycogen	Deuterium concentration of glycogen	Deuterium concentration of body water
	atom % excess	%	*atom % excess*	*atom % excess*
L9	52.2	0.96	0.25	0.02
L10	76.4	0.96	0.31	

evaporated to dryness. Hydrogen for mass spectrometric analysis was liberated by heating the sodium formate in an evacuated break-seal tube at 400° for 1 hour.

A second product of this oxidation is D′-methoxy-D-hydroxymethylglycoldialdehyde. This was oxidized by the method of Boothroyd *et al.* to the corresponding dicarboxylic acid which was then isolated as strontium D′-methoxy-D-hydroxymethyldiglycolatedihydrate. For deuterium analysis the anhydrous salt was prepared by heating the dihydrate in a vacuum at 100° over P_2O_5 for 24 hours.

Periodate Oxidation of Potassium Gluconate—Formaldehyde with the hydrogen atoms in position 6 was obtained as the dimedon derivative after oxidation of potassium gluconate with periodate under the conditions described by Reeves (5). The amounts of the dimedon-formaldehyde complex obtained in this way from potassium gluconate of Experiments L9 and L10 were equivalent to 91 and 95 per cent of theory, respectively. Both samples melted instantly at 189° (uncorrected).

Preparation of Glucosazone—The procedure of Vogel (6) was adopted for the preparation of glucosazone.

RESULTS

Column 3 of Table II shows the observed deuterium concentrations of the derivatives prepared from the liver glycogen obtained in Experiment L9. The deuterium concentrations of the carbon-bound hydrogen atoms of the original glucose remaining in the derivatives (positions are also shown) are listed in Column 1. These data were calculated by correcting the observed isotope concentration of the derivative for dilution by the normal hydrogen atoms present in the derivative. For example, from the deuterium concentration of the α-methyl glucoside, 0.19 atom per cent excess, and the total number of hydrogen atoms in the derivative (Column 2), the deuterium concentration of the 7 carbon-bound hydrogen atoms of the original glucose is computed to be $0.19 \times \frac{14}{7} = 0.38$ atom per cent excess.

The concentrations listed in Table II, Column 1, are used to calculate the apparent deuterium concentrations of the individual hydrogen atoms of the labeled glucose. It can be noted that relatively small differences between large numbers sometimes appear in the calculations, a circumstance which, based on an analytical error of ± 0.01 atom per cent, leads to an error in the final result of approximately ± 0.2 atom per cent.

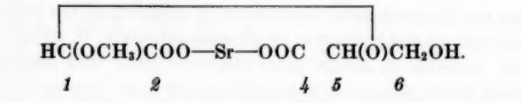
Calculations of Deuterium Concentrations of Individual Hydrogen Atoms of Glucose—The deuterium concentration of the carbon-bound hydrogen atom in position 1 of glucose is obtained from the corrected deuterium concentrations of the glucoside and gluconate (Table II, Column I); concentration at $C_1 = 7 \times 0.38 - 6 \times 0.44 = -0.02$ atom per cent excess.

In Experiment L10 the deuterium concentration of the potassium gluconate was 0.29 atom per cent excess and of the glycogen, 0.31 atom per cent excess. The corrected concentrations are 0.53 and 0.44 atom per cent excess, respectively. The deuterium concentration at C_1 is thus $7 \times 0.44 - 6 \times 0.53 = -0.10$ atom per cent excess. The computation indicates that no deuterium is present in position 1 of glucose.

In a similar manner the deuterium concentration of the hydrogen atom at C_2 may be estimated from the corrected deuterium concentrations of the glucoside and glucosazone, *i.e.* concentration at $C_2 = 7 \times 0.38 - 6 \times 0.46 = -0.10$ atom per cent excess. This result indicates that deuterium is absent also from the 2 position of glucose.

The deuterium concentration of the hydrogen atom bound to C_3 was determined directly by analysis of the molecular hydrogen yielded by thermal decomposition of the formate arising from position 3. As noted in Table II, this was found to be 0.04 atom per cent excess, a value which lies outside the limits of experimental error.

The structure of strontium D′-methoxy-D-hydroxymethyldiglycolate is as follows:



The numerals in italics refer to the positions of the carbon atoms in the original glucose. Attention is called to the absence of hydrogen bound to carbon atoms corresponding to positions 2 and 4 of glucose. The diglycolate thus contains only the hydrogen atoms of glucose present in positions 1, 5, and 6. The concentration of deuterium in positions 2, 3, and 4 of glucose can

TABLE II

Deuterium concentrations of derivatives prepared from labeled glycogen

Compound	I Carbon-bound H atoms of original glucose in derivative		II Total number of H atoms in derivative	III Observed deuterium concentration of derivative
	Position	Deuterium concentration*		
		atom % excess		*atom % excess*
α-Methylglucoside	1, 2, 3, 4, 5, 6, 6	0.38	14	0.19
Potassium gluconate	2, 3, 4, 5, 6, 6	0.44	11	0.24
Glucosazone	1, 3, 4, 5, 6, 6	0.46	22	0.13
Sodium formate from periodate oxidation of α-methyl glucoside	3	0.04	1	0.04
Sodium formate from periodate oxidation of potassium gluconate	2, 3, 4, 5	0.54	4	0.54
Strontium D′-methoxy-D-hydroxymethyldiglycolate	1, 5, 6, 6	0.18	8	0.09
Dimedon-formaldehyde	6, 6	0.44	22	0.04

* See the text.

therefore be calculated from the corrected deuterium concentrations of the glucoside and diglycolate. This is

$$\frac{7 \times 0.37 - 4 \times 0.18}{3} = 0.62$$

atom per cent excess. From the deuterium concentrations of the hydrogen atoms in positions 2 and 3 it can be seen that the concentration in position 4 must be $3 \times 0.62 - 0 - 0.04 = 1.82$ atom per cent excess.

The concentration of deuterium in position 5 can be calculated from those in positions 1 and 6, provided the concentrations in positions 1, 5, and 6 are known. The value in position 6 can be obtained by analysis of the dimedon derivative of the formaldehyde yielded upon periodate oxidation of potassium gluconate. The analyses of the dimedon-formaldehyde derivative obtained in Experiment L9 were performed in triplicate with a standard error of ± 0.006 atom per cent. The dimedon-formaldehyde compound prepared in the same manner from material obtained in Experiment L10 contained 0.05 ± 0.005 atom per cent excess deuterium (two determinations). There would thus seem to be no doubt that deuterium is present in relatively high concentration (0.44 and 0.55 atom per cent excess, respectively) in the hydrogen atoms located at position 6 of the glucose formed in Experiments L9 and L10.

The concentration of deuterium in position 5 can now be calculated from the deuterium concentration of hydrogen in positions 1, 5, and 6 (0.18 atom per cent excess) and the deuterium concentrations of the hydrogen atoms in positions 1 (0 atom per cent excess) and 6 (0.44 atom per cent excess), *i.e.* concentration in position $5 = 4 \times 0.18 - 0 - 2 \times 0.44 = -0.16$ atom per cent excess. This result indicates that there is no excess deuterium in the 5 position.

DISCUSSION

The results presented above show that after administering DL-α-deuteriolactate to fasting rats, highly significant amounts of deuterium appear in positions 4 and 6 of glucose derived from the liver glycogen. Inasmuch as the transfer of hydrogen between DPN and lactate, catalyzed by L-lactic dehydrogenase and between DPN and Gl-3-P, catalyzed by triose phosphate dehydrogenase, is direct (7, 8), coupling of the oxidation of L-α-deuteriolactate to the reduction of 1,3-diphosphoglycerate may be expected to lead to the formation of Gl-3-P which contains deuterium in the aldehyde group. The participation of this product in reactions catalyzed by triose phosphate isomerase and aldolase may, in turn, be expected to yield fructose-DP labeled in positions 3 and 4, and thus in subsequent reactions to form glucose similarly labeled, provided, of course, that at the same time the substituted deuterium atoms are not replaced by exchange with hydrogen of the body water.

When DHAP in a medium containing tritium oxide is acted upon by triose phosphate isomerase or by aldolase, 1 atom of carbon-bound tritium appears in the primary carbinol group (9, 10). The enzymes are absolutely stereospecific, acting upon hydrogen atoms of opposite configuration (10, 11). It can be presumed that in the presence of both enzymes in normal water deuterium initially introduced into the aldehyde group of Gl-3-P would be removed from the DHAP, subsequently formed, by exchange. The presence of a small amount of deuterium in position 3 suggests that the exchange reactions involving DHAP did not go to completion before the condensation of the triose phosphates.

Formation of unlabeled Gl-3-P must also be presumed to take place by the action of isomerase on unlabeled DHAP. The present data show that the rate of this reaction *in vivo* is not so great as to result in dilution of deuterium beyond detection in the liver glycogen. This suggests that the rate of formation of Gl-3-P from DHAP is slow in relation to the rate of formation of DHAP from Gl-3-P.

Notwithstanding the fact that the concentration of deuterium in position 4 of glucose indicates the net result of concurrent processes of labeling and unlabeling, it can be noted that in Experiment L9 almost 4 per cent $\left(1.83 \times \frac{100}{52.2}\right)$ of the hydrogen atoms in that position were derived from the α-hydrogen atom of the administered lactate.

To account for the presence of deuterium in position 6, the possibility of equilibration of 1-deuterio-Gl-3-P with a 3-carbon symmetrical intermediate, *i.e.* glycerol, suggests itself. However, this mechanism does not satisfactorily account for the present data. As discussed above, DHAP formed from 1-deuterio-Gl-3-P seems to be almost completely unlabeled. Thus in the sequence of reactions, 1-deuterio-Gl-3-P ↔ DHAP ↔ α-glycerophosphate ↔ glycerol, it can be presumed that the glycerol product would contain little, if any, deuterium. Furthermore, the stereospecificities of the enzyme-catalyzed transformations shown above are such that even were deuterium present in DHAP, thus permitting the formation of labeled glycerol, reversal of the process would yield 1-deuterio-Gl-3-P and not 3-deuterio-Gl-3-P (12–15).

The present results are plausibly accounted for by assuming the operation *in vivo* of the following sequence of reactions:

$$CH_3CD(OH)COOH + TPN^+ \xrightleftharpoons{\text{lactic dehydrogenase}} CH_3COCOOH + TPND + H^+ \quad (1)$$

$$CH_3COCOOH + CO_2 + TPND + H^+ \xrightleftharpoons{\text{malic enzyme}} HOOCCD(OH)CH_2COOH + TPN^+ \quad (2)$$

$$HOOCCD(OH)CH_2COOH \xrightleftharpoons{\text{fumarase}} HOOCCD{=}CHCOOH \quad (3)$$

$$HOOCCD{=}CHCOOH \xrightleftharpoons{\text{fumarase}} HOOCCDHCH(OH)COOH \quad (4)$$

$$HOOCCDHCH(OH)COOH + DPN^+ \xrightleftharpoons{\text{malic dehydrogenase}} HOOCCHDCOCOOH + DPNH + H^+ \quad (5)$$

$$HOOCCDHCOCOOH + ITP \xrightleftharpoons{\text{oxalacetate carboxylase}} CHD{=}C(OPO_3H_2)COOH + IDP \quad (6)$$

This scheme is in accord with well established knowledge of the participation of C_4 symmetrical intermediates in the synthesis of liver glycogen from lactate.

It will be noted in Reaction 1 that TPN is indicated as acting as the coenzyme of lactic dehydrogenase. This, rather than the assumption of the participation of a transhydrogenase, is based upon the experimental results of Mehler *et al.* (16), which demonstrate nonspecific utilization of TPN as coenzyme in the coupling of lactate oxidation and malate synthesis. More recently, Navazio *et al.* (17) observed oxidation of TPNH by pyruvate with the formation of lactate in a soluble extract of rat liver homogenate.

The incorporation of deuterium in liver glycogen after the administration of DL-2,3-deuteriomalate was observed to be approximately one-third of that noted after administration of DL-α-deuteriolactate (1). This result, in the light of the present studies, suggests that the hydrogen of exogenous malate is not utilized as efficiently as that of endogenous malate in the reductive synthesis of hexose. This hypothesis was tested in recent preliminary experiments by studies of the incorporation of deuterium in liver glycogen formed from administered α-α′-deuteriofumarate. The deuterium concentration of the glycogen was found to be comparable to that observed after administration of α-deuteriolactate. The glucose obtained upon hydrolysis of the glycogen was labeled in position 6 and unlabeled in position 1. Only half of the deuterium in the molecule was accounted for by that in position 6. Degradative studies are now in progress for the purpose of disclosing the positions occupied by the remaining deuterium.

3-Deuteriophosphopyruvate, presumably formed as a product of the reactions depicted above, might be expected to yield fructose-DP labeled in both the 1 and 6 positions and subsequently, fructose-6-P similarly labeled. In the transformation of fructose-6-P to glucose-6-P by the action of hexose phosphate isomerase 1 atom of hydrogen is stereospecifically removed from the primary carbinol group (18). The present data indicate that a hydrogen atom is similarly removed *in vivo* because, had the loss of hydrogen from this position taken place at random, the deuterium concentration in position 1 of glucose would have been observed to be approximately one-half of that in position 6. It must be supposed also that both β-hydrogen atoms of malate, oxalacetate, and phosphopyruvate are and remain sterically distinct, because any process leading to random distribution of the β-hydrogen atoms would lead ultimately to an apparent random distribution of deuterium in the 1 position of the fructose phosphates. One process which would lead to the loss of steric specificity of the β-hydrogen atoms of oxalacetate is enolization. In this connection it is significant that the enol form of oxalacetate has been ruled out as a product of the oxaloacetate carboxylase reaction (19) and as the substrate and product of the action of malic dehydrogenase (20). These considerations further suggest that in the present studies, only one of the two positions at C-6 of glucose is substituted with deuterium. The deuterium concentration of the hydrogen in this position is thus estimated to be $2 \times 0.44 = 0.88$ atom per cent excess (Experiment L9), and $2 \times 0.55 = 1.10$ atom per cent excess (Experiment L10). The deuterium concentration in position 6 would thus seem to be approximately one-half of that in position 4 (0.88/1.82).

SUMMARY

Glycogen, containing an excess of stably bound deuterium, was isolated from the livers of rats which had received DL-α-deuteriolactate. Glucose obtained by hydrolysis of the labeled glycogen was subjected to degradative procedures in order to locate the deuterium in the molecule. Little or no deuterium was found in positions 1, 2, 3, and 5. The highest concentration of deuterium was in position 4. Deuterium was also observed to be present in relatively high concentration in position 6. These results are interpreted to indicate that the α-hydrogen atom of lactate is involved in the reductive synthesis of triose phosphate and in the reductive formation of malate.

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Ribitol Dehydrogenase

I. PURIFICATION AND PROPERTIES OF THE ENZYME*

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(Received for publication, July 24, 1958)

The existence of DPN-dependent polyol dehydrogenases in guinea pig mitochondria has been demonstrated by Hollmann and Touster (1). The enzyme preparation is capable of catalyzing a number of hexitol-ketohexose and pentitol-ketopentose transformations. It remains to be ascertained whether the dehydrogenase represents a truly nonspecific enzymatic action or whether the mitochondrial preparation contains a number of specific polyol dehydrogenases. Wood and Tai (2) reported the presence of a DPNH specific ribulose reductase from *Aerobacter aerogenes* strain PRL R3 which converts ribulose to ribitol.[1]

The purpose of this paper is to report studies on the purification and properties of ribitol dehydrogenase from *A. aerogenes* (ATCC 9621), which catalyzes the following reaction: ribitol + $DPN^+ \rightleftarrows$ D-ribulose + DPNH + H^+.

EXPERIMENTAL

Preparation of Cell-Free Extract—A. aerogenes (ATCC 9621) was grown on a ribitol and inorganic salt medium (3) for 24 hours at 37° with shaking. The cells were collected by centrifugation at 3°, washed with cold 0.1 M Tris[2] buffer, pH 7.4, and recentrifuged. This operation was repeated three times. Finally, 10 gm. of cells (wet weight) were suspended in 30 ml. of 0.1 M Tris buffer, pH 7.4, containing 30 gm. of glass beads and exposed to a 9 kc. Raytheon sonic oscillator for 30 minutes.

Materials—DPN, TPN, and ATP were products of the Pabst Laboratories. DPNH was a product of the Sigma Chemical Company. The sugars unless otherwise specified were obtained from Nutritional Biochemicals Corporation.

Ribitol-1-phosphate was prepared by the reduction of D-ribose-5-phosphate with H_2 (1500 pounds per sq. in.) in the presence of Raney nickel at 50°. After removal of the catalyst the pH of the solution was adjusted to neutrality and the phosphate content of the ribitol-1-phosphate determined after HNO_3-H_2SO_4 hydrolysis (4). The product gave a negative test for ribose (5), reducing sugar (Fehling's test) and free phosphate (4).

D-Ribulose was prepared by boiling D-arabinose in dry pyridine (6). *o*-Nitrophenyl hydrazone derivatives of D- and L-ribulose were gifts of Dr. P. Smyrniotis. D- and L-ribulose were generously donated by Dr. W. A. Wood. The concentration of ribulose from benzaldehyde-decomposed *o*-nitrophenyl hydrazone derivatives (6) was determined with periodic acid (7) with D-fructose as a standard.

Yeast alcohol dehydrogenase was bought from the Worthington Biochemical Corporation.

Determinations—Spectrophotometric measurements were made with a Beckman model DU spectrophotometer. Ribulose was determined by the method of Dische and Borenfreund (8). DPN, TPN, and DPNH were estimated from their absorption spectra (9). DPN was determined in the presence of DPNH according to the procedure of Colowick *et al.* (10) with the use of yeast alcohol dehydrogenase. Protein was determined by the method of Warburg and Christian (11).

Ribulose Synthesis—The synthesis of ribulose from ribitol and DPN was measured in several ways. Before the product of ribitol oxidation was identified as ribulose, the following reaction mixture (Assay I) was used: 2 μmoles of DPN, 2 μmoles of ribitol, 400 μmoles of Tris, pH 7.4, and enzyme in a total volume of 0.6 ml. Incubation was carried out for 20 minutes at 37°. 2.0 ml. of 3.5 per cent $HClO_4$ were added to terminate the reaction and 100 mg. of acid-washed Norit were then added. After standing for 30 minutes the Norit was removed by filtration and an aliquot of the supernatant solution was analyzed either by the $FeCl_3$-orcinol test (5) or directly for ribulose (8). Boiled enzyme preparations were used as controls.

DPNH formation during the steps of enzyme purification (Assay II) was measured in reaction mixtures containing 4 μmoles of DPN, 4 μmoles of ribitol, 400 μmoles of Tris, pH 7.4, and enzyme in a total volume of 2.3 ml. Control tubes contained boiled enzyme. A unit of enzyme activity was defined as the amount necessary to cause an increase in optical density of 0.100 at 340 mμ in 2 minutes at 28°.

RESULTS

Purification of Ribitol Dehydrogenase—Purification of the enzyme was carried out at 3° unless otherwise stated. The pH of all buffers and reagents was adjusted at room temperature and the solutions were then cooled to 3°. Activity yields in excess of 100 per cent are due primarily to the removal of DPNH oxidase activity.

Streptomycin Treatment—To 30 ml. of cell-free extract obtained from 10 gm. of *A. aerogenes* were added 35 ml. of 0.1 M

* This study was supported in part by a research grant (A-1678) from the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service.

[1] The author is indebted to Dr. W. A. Wood, University of Illinois, for calling his attention to the abstract on the existence of ribulose reductase (2).

[2] The abbreviation used is: Tris, tris(hydroxymethyl)aminomethane chloride.

TABLE I

Purification of ribitol dehydrogenase

Enzyme fraction	Total units*	Total protein	Specific activity
		mg.	*units/mg. protein*
Cell-free extract	1460	2130	0.69
Streptomyocin	2610	1190	2.2
Low pH	3090	630	4.9
Ammonium sulfate	2590	214	12.1
Gel treatment	1690	45	37.5

* The low recovery of activity in the cruder preparations is believed to be due to DPNH oxidase activity.

Tris buffer, pH 7.4. 13 ml. of 5 per cent streptomyocin sulfate (Chas. Pfizer and Company) were slowly added to the extract with stirring. After standing for 10 minutes the precipitate was removed by centrifugation at 10,000 × *g* for 10 minutes.

Low pH Treatment—The pH of the supernatant solution obtained after streptomyocin treatment was adjusted to 6.2 with 0.1 N acetic acid. It was then transferred to a water bath and stirred vigorously for 20 minutes at 40°. The resultant precipitate obtained by centrifugation at 10,000 × *g* for 10 minutes was discarded.

Ammonium Sulfate Precipitation—The pH of the supernatant solution was adjusted to 7.15 by the slow addition of 0.1 N NaOH. Enough solid ammonium sulfate was added with constant stirring to the solution to bring the saturation to 35 per cent. After standing for 5 minutes the precipitated protein was collected by centrifugation (10,000 × *g* for 10 minutes) and discarded. Additional ammonium sulfate was added to the supernatant fluid to bring the saturation to 50 per cent. After standing for 15 minutes the precipitate was collected by centrifugation (10,000 × *g* for 10 minutes). The precipitate was dissolved in 50 ml. of 0.1 M Tris buffer, pH 7.4, and then was dialyzed against running 0.1 M Tris buffer, pH 7.4, in a rocking dialyzer for 4 hours.

Calcium Phosphate Gel Treatment—The following operations were carried out at room temperature. 6.0 ml. of calcium phosphate gel (23.7 mg. of solids per ml.) (12) were added with stirring to 30 ml. of the dialyzed supernatant fluid (4.5 mg. of protein per ml.). The pH of the suspension was adjusted to 6.0 with 0.1 N acetic acid, the suspension being allowed to stand for 10 minutes, and the gel was removed by centrifugation (2500 × *g* for 5 minutes). An additional 20 ml. of gel were added to the supernatant solution and the pH adjusted to 6.2 with 0.1 N acetic acid. After standing 10 minutes the gel was removed by centrifugation and the supernatant solution retained. The enzyme was adsorbed on the gel by repeating the last step with the use of 20 ml. of gel.

The gel was suspended in 20 ml. of 1 M phosphate buffer, pH 8.0, the suspension allowed to stand for 30 minutes, and centrifuged at 2500 × *g* for 10 minutes. This last procedure was repeated and the supernatant fluids obtained were pooled. The solution was finally dialyzed against running 0.1 M Tris, pH 7.4, in a rocking dialyzer for 4 hours at 3°.

The purified enzyme retained its activity during several weeks of storage at 3°. Recovery data for the purification of the enzyme are presented in Table I.

Identification of Reaction Products—Purified ribitol dehydrogenase in the presence of its substrates (Assay I) catalyzes the reduction of DPN to DPNH (identified by its absorption spectrum (9)) and ribulose. The latter product was identified as follows: by its positive cysteine-carbazole reaction within 10 minutes (8) and its $FeCl_3$-orcinol test ratio $E_{540}:E_{570}$ of 0.86 (13). Further substantiation of the formation of ribulose as the product of ribitol oxidation was obtained by paper chromatography of an aliquot of Assay I solution in *n*-butanol-H_2O according to the method of Cohen (14). An authentic sample of D-ribulose was used as a standard.

Specificity of Reaction—It was found that TPN was not capable of replacing DPN in Assay II. Ribitol-1-phosphate exhibited 60 per cent of the activity observed with ribitol in cell-free extracts. Purified preparations of the enzyme did not, however, utilize ribitol-1-phosphate as a substrate. This suggests that the phosphorylated compound had been hydrolyzed to free ribitol by an enzyme present in the extract. In view of the work of Hollmann and Touster (1) on pig liver mitochondria polyol dehydrogenases, a number of compounds including D-sorbitol, dulcitol and D-xylitol were tested, but could not replace ribitol in Assay II. Furthermore, L-ribulose, D-fructose, and D- and L-arabinose were not capable of replacing D-ribulose in the reverse reaction (see below).

pH Dependence—Fig. 1 shows the velocity of ribitol dehydrogenase as a function of pH. It can be seen from the curve that the enzyme exhibits a sharp maximal pH from 10 to 10.5.

Stoichiometry of Reaction—When DPNH and D-ribulose were substituted for DPN and ribitol (Assay II), it was found that the reaction velocity in the reverse direction was much greater than that in the forward direction at neutral pH. Studies with the purified enzyme (specific activity, 37.5) indicated that equivalent amounts of D-ribulose and DPNH were formed in the forward direction. Similarly, equivalent amounts of these compounds disappeared in the reverse reaction. In Table II are shown data obtained from a typical balance study.

Equilibrium Studies—It was possible from the stoichiometry of the reaction to determine the equilibrium constant, *K*, of the reaction by merely determining the appearance of ribulose and DPNH (forward direction) or the disappearance of these compounds (reverse direction). Tables III and IV illustrate the equilibrium constants obtained at various pH values. It was found that after equilibrium attainment the concentration of DPNH (measured spectrophotometrically) could be appropriately shifted by the addition of either sugar. The average *K* was calculated to be 1.49×10^{-10}. Hollmann and Touster (1) report a *K* of 8.58×10^{-11} for an analogous reaction involv-

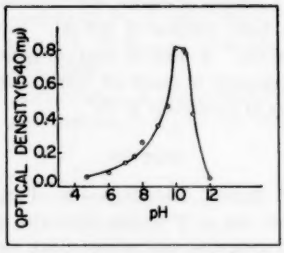

FIG. 1. Graph of velocity *versus* pH values. The reaction mixture contained 2 μmoles of ribitol, 2 μmoles of DPN, 150 μmoles of phosphate buffer and 3.3 units of ribitol dehydrogenase (specific activity 37.5) in a total volume of 0.60 ml. The reaction mixtures were incubated for 40 minutes at 37°. Ribulose color in the cysteine-carbazole reaction (8) is recorded on the ordinate.

TABLE II

Stoichiometry of ribitol dehydrogenase

The reaction mixture contained: 6.10 μmoles of D-ribulose, 3.35 μmoles of DPNH, 300 μmoles of Tris buffer, pH 7.4, and enzyme (6.5 units; specific activity, 37.5) in a total volume of 0.70 ml. Incubation was for 30 minutes at 37°. The reaction was terminated by boiling the mixture at 100° for 2 minutes. Boiled enzyme served as the control. Assays were made as follows after dilution of the reaction mixture with H_2O: ribulose by the cysteine-carbazole method (8), DPNH at E_{m340}, pH 7.5 (6.22×10^3), and DPN with alcohol dehydrogenase (10).

	0 min.	30 min.	Δ
	μmoles	*μmoles*	*μmoles*
D-Ribulose.....	6.10	2.81	3.29
DPNH.....	3.35	0.19	3.14
Ribitol.....	0.00	(3.29)*	3.29
DPN.....	0.00	3.20	3.20

* Estimated from the disappearance of D-ribulose.

TABLE III

Equilibrium starting with ribitol and DPN

The reaction mixtures also contained 300 μmoles of Tris buffer and 6.5 units of enzyme (specific activity 37.5). The flasks were incubated for 3 hours. Boiled enzyme preparations served as controls.

pH values	Temperature	Added initially		Equilibrium values				$K \times 10^{-10}$
		DPN	Ribitol	D-Ribulose	DPNH	Ribitol*	DPN*	
	degrees	*μmoles*	*μmoles*	*μmoles*	*μmoles*	*μmoles*	*μmoles*	
7.4	37	2.20	2.00	0.11	0.10	1.89	2.10	1.11
8.0	37	2.91	2.00	0.19	0.22	1.81	2.69	0.90
8.5	37	2.20	2.00	0.30	0.33	1.70	1.87	0.99

* These values were estimated from the appearance of D-ribulose and DPNH.

TABLE IV

Equilibrium starting with D-ribulose and DPNH

The reaction mixtures also contained 300 μmoles of Tris buffer and 6.5 units of enzyme (specific activity 37.5). The flasks were incubated for 3 hours. Boiled enzyme preparations served as controls.

pH values	Temperature	Added initially		Equilibrium values				$K \times 10^{-10}$
		DPNH	D-Ribulose	D-Ribulose	DPNH	Ribitol*	DPN*	
	degrees	*μmoles*	*μmoles*	*μmoles*	*μmoles*	*μmoles*	*μmoles*	
7.4	37	1.64	1.56	0.19	0.04	1.37	1.60	1.39
8.0	37	1.64	2.02	0.60	0.04	1.42	1.60	1.06
8.5	37	1.64	1.87	0.52	0.36	1.35	1.28	3.41

* These values were estimated from the disappearance of D-ribulose and DPNH.

ing the xylitol-L-xylulose enzyme which utilizes TPN. Wood[3] has suggested that the favorable equilibrium might be used as an analytical tool for the determination of D-ribulose-5-phosphate after dephosphorylation.

Kinetic Studies—With the use of Assay II and enzyme, the reaction velocity as a function of enzyme concentration was found to be linear. These data are shown in Fig. 2. Concentrations of enzyme in excess of those shown in Fig. 2 resulted in a decreased velocity, presumably caused either by accumulation of products or depletion of substrates.

[3] Dr. W. A. Wood, personal communication.

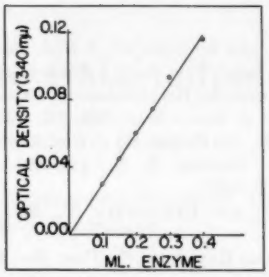

FIG. 2. Graph of velocity *versus* enzyme concentration. The reaction mixture contained 4 μmoles of ribitol, 4 μmoles of DPN, 400 μmoles of Tris buffer, pH 7.4, and ribitol dehydrogenase (46 μg. of protein per ml., specific activity 20) in a total volume of 2.30 ml. Incubation was carried out for 5 minutes at 28.5°.

TABLE V

Apparent Michaelis constants for ribitol dehydrogenase

Molarity of second substrate	K_m
DPNH = 1.4×10^{-3}	D-Ribulose = 1.4×10^{-2} M
D-Ribulose = 5.4×10^{-3}	DPNH = 3.2×10^{-4} M
Ribitol = 4.5×10^{-2}	DPN = 1.5×10^{-3} M
DPN = 4.4×10^{-3}	Ribitol = 8.5×10^{-2} M

The apparent Michaelis constants (K_m) for ribitol dehydrogenase were determined for the substrates in both the forward and reverse reactions. These constants represent saturation or near-saturation levels of the nonvaried substrate. The data obtained from these studies are presented in Table V.

DISCUSSION

The results presented in these investigations, along with the observations of Wood and Tai (2), suggest that the pathway for ribitol metabolism in *A. aerogenes* is as shown in Reaction 1.

$$\text{Ribitol} \rightarrow \text{D-ribulose} \xrightarrow{\text{ATP} \rightarrow \text{ADP}} \text{D-ribulose-5-phosphate}$$

REACTION 1

Although the ribitol-D-ribulose transformation hardly favors D-ribulose synthesis (as indicated from the equilibrium constant), coupling of this reaction to one involving ATP utilization in the presence of D-ribulokinase would produce an over-all sequence that is feasible thermodynamically.

SUMMARY

1. Ribitol dehydrogenase from *Aerobacter aerogenes* has been purified 54-fold.

2. The enzyme catalyzes the following reaction: ribitol + $DPN^+ \rightleftarrows$ D-ribulose + DPNH + H^+.

3. The enzyme seems to be specific for the substrates indicated.

4. The average equilibrium constant at three different pH values was found to be 1.49×10^{-10}.

5. The properties of the purified enzyme are described.

Acknowledgment—The author wishes to express his thanks to Dr. John W. Vennes for providing the *A. aerogenes*.

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The Incorporation of Galactose into Galactolipides

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(Received for publication, June 13, 1958)

Since the pioneering studies of Thudichum (1), a number of investigations have been undertaken to elucidate the composition and to determine the quantity of the sugar-containing lipides in animal tissues. A number of glycolipides have been described, *e.g.* cerebrosides (1), cerebron sulfate (2), polycerebrosides (3), gangliosides (4, 5), and strandin (6–8). Within the last few years studies of the metabolism of these lipides have been successfully undertaken. Meltzer (9) and Weiss (10) showed that the perfusion of brain with radioactive fatty acids causes the incorporation of labeled carbon into cerebral lipides. Recently, Radin *et al.* (11) Moser and Karnovsky (12, 13), and Burton *et al.* (14–16) have demonstrated that the administration of C^{14}-labeled hexoses to the rat and mouse results in the radioactive labeling of brain glycolipides.

The present communication reports the details of experiments which show that the incorporation *in vivo* of glucose and galactose into brain lipides of the rat is a function of age, occurring most rapidly between 10 and 20 days *post partum*. Further experiments conducted *in vitro* indicate that uridine diphosphogalactose is involved in the incorporation of galactose into neutral glycolipides (probably cerebrosides), and the reaction is catalyzed by enzymes present in microsomal preparations of rat brain tissue.

EXPERIMENTAL

Male albino rats raised in the National Institutes of Health colony of the Holtzman strain were used in all the experiments reported. The labeled hexoses were administered as aqueous solutions intraperitoneally. The animals were killed by cervical fracture, the whole brains rapidly removed and immediately processed by either Procedure I or II described below.

Procedure I—The tissue was homogenized in cold 1 M glucose or galactose solution and then centrifuged. The supernatant fluid was decanted and the residue washed with 1 M glucose or galactose. The supernatant layers were combined and designated as the *aqueous fraction*. The precipitate was homogenized twice in acetone and centrifuged. The acetone supernatant fluids were combined and called the *acetone fractions*. The precipitate was then homogenized in chloroform-methanol, 2:1, and centrifuged. The residue was discarded. The neutral glycolipide fraction was obtained in the manner described in Procedure II by use of the chloroform-methanol, 2:1, solution.

Procedure II—The excised tissue was rapidly weighed and immediately homogenized in 20 volumes of CM.[1] The mixture was heated to boiling and then allowed to cool to room temperature. The CM mixture, including the precipitate, was passed over a column composed of a mixed-bed of Nalcite SBR-8 (Dowex 1) (National Aluminate Corporation) and Nalcite HCR-8 (Dowex 50) (National Aluminate Corporation) layered with Florisil (Floridin Company) essentially as described by Radin *et al.* (17). The precipitate and column were washed with 50 ml. of CM. The combined eluates were taken to dryness by passing an air stream across the receiving beaker. The lipide residue was dissolved in 5 ml. of CM and transferred with 5 ml. of CM washings to a glass-stoppered, tapered 50 ml. centrifuge tube. An equal volume (10 ml.) of chloroform-saturated methanol-water, 1:1, solution was added to the tube; the tube was stoppered and shaken mechanically for 30 minutes. The two phases were separated by low-speed centrifugation. The aqueous layer was carefully removed and replaced with another 10 ml. of the methanol-water solution. The tubes were again shaken and centrifuged. During the washing procedure, the volume of the chloroform phase decreased as a result of the greater solubility of methanol in water. As the methanol concentration decreased in the chloroform phase, a white precipitate developed. In removal of the aqueous phase all of the precipitate at the interface was retained with the chloroform phase. The chloroform phase and precipitate were washed five times at which time the aqueous phase contained no radioactivity. This step was found to be necessary, particularly in the experiments conducted *in vitro* with galactose-1-C^{14} and glucose-U-C^{14}, since the hexoses are not retained by the components of the mixed-bed column. After the final wash, the precipitate was dissolved by adding methanol. An aliquot of the CM solution was plated on metal planchets and the radioactivity determined.

After chromatography on the mixed-bed column, the glycolipide fraction (5 μmoles) was dissolved in 5 ml. of CM and decolorized with 5 to 10 mg. of Norit A (American Norit Company). This step was necessary particularly when Procedure II was used; apparently some neutral lipides, which were removed by the *acetone* step in Procedure I, caused the appearance of an interfering brown color in the colorimetric determination. The glycolipide content of a 1 ml. aliquot of the CM solution was estimated by the method described by Radin *et al.* (17).

Glucose-U-C^{14} (1 mc. per mmole) was obtained from either the National Bureau of Standards or the Volk Radio Chemical Company. Galactose-1-C^{14} (0.3 mc. per mmole or 0.25 mc. per mmole) was prepared by the National Bureau of Standards. Galactose(-1-C^{14})-1-phosphate was prepared from galactose-1-C^{14} by phosphorylation with ATP and galactokinase (18). The galactose(-1-C^{14})-1-phosphate was isolated as the barium salt.

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[1] The abbreviation used is: CM, solvent mixture of chloroform-methanol (2:1, volume for volume).

UDP-galactose-1-C^{14} was prepared from galactose(-1-C^{14})-1-phosphate by the exchange reaction with UDP-glucose catalyzed by galactose-1-phosphate uridyl transferase. The excess UDP-glucose was oxidized with UDP-glucose dehydrogenase and the UDP glucuronic acid was separated from the UDP-galactose by paper chromatography (19, 20).[2]

Methanolysis of Glycolipides—The glycolipides were refluxed (88–92°) for 100 minutes in chloroform-methanol, 2:1, which contained hydrochloric acid at a final concentration of 0.6 M. After cooling, the cleavage products were partitioned between water and chloroform. The emulsion formed by shaking the aqueous and organic phases was broken by centrifugation. The two phases were separated and the radioactivity and hexose content of each phase was determined.

RESULTS

Incorporation of Galactose-C^{14}—The distribution *in vivo*, of the radioactivity recovered in the water-soluble, acetone-soluble, and neutral glycolipide fractions of rat brain and liver tissue is illustrated by the data presented in Table I. Even though the total radioactivity in the liver decreased with time, the relative activity of the three fractions remained essentially constant for liver tissue. More than 90 per cent of the radioactivity was present in the aqueous fraction, a few per cent in the acetone-soluble fraction, and less than 1 per cent in the neutral glycolipide fraction. A different pattern was found for the distribution of the C^{14} in brain tissue. The proportion of the radioactivity of the aqueous fraction obtained from brain tissue decreased from about 96 to 98 per cent at 2 hours to 83 to 87 per cent at 6 to 8 hours after the injection of the labeled sugar. The most striking change is noted in the neutral glycolipide fraction where the incorporated radioactivity increased from 1 to 2 per cent at 2 hours to 10 to 13 per cent at 8 hours after the administration of galactose-C^{14}.

Similar results were obtained with the intraperitoneal injection of glucose-U-C^{14} (Table II). The amount of the radioactivity in the aqueous fraction decreased with time whereas that of the acetone fraction increased. The proportion of the radioactivity in the chloroform-methanol fraction, which contained lipides in addition to the neutral glycolipides, increased from about 10 to about 30 per cent, and the neutral glycolipide fraction increased from 2 to about 8 per cent during the interval from 2 to 8 hours after administration of the labeled glucose. There is, therefore, considerable incorporation of radioactivity from both glucose-U-C^{14} and galactose-1-C^{14} into the neutral glycolipide fraction of brain tissue and only negligible incorporation of radioactivity into the neutral glycolipide fraction of liver tissue.

Time Course of Incorporation in Vivo of Glucose-U-C^{14} into Brain Tissue Neutral Glycolipide—Glucose-U-C^{14} was administered to young male rats which were killed at intervals up to 24 hours after the injection of glucose-C^{14}. The incorporation of radioactivity into the neutral glycolipide (cerebroside) fraction of rat brain tissue increased with time until a maximum occurred at about 6 hours. Thereafter, the radioactivity in this fraction decreased and the value observed 24 hours after the administration of the labeled hexose was about one-third of the maximal level (Fig. 1). Experiments with galactose-1-C^{14} gave identical results.

Effect of Age upon Incorporation of Galactose-1-C^{14}—Male rats were killed 8 hours after the intraperitoneal administration of galactose-1-C^{14}. It may be seen that animals younger than 7 days *post partum* incorporated very little of the C^{14}-hexoses into the cerebroside fraction of brain tissue (Fig. 2). Animals older than 20 to 25 days showed a markedly reduced ability to incorporate radioactive galactose. The optimal age of rats for the incorporation of galactose-1-C^{14} into the cerebroside fraction was between 10 and 18 days. All subsequent experiments were therefore performed with male rats within the age range of 14 to 16 days *post partum*. It has been observed by Moser and Karnovsky (12, 13) that newborn mice can incorporate glucose-C^{14} into a cerebroside fraction. However, extensive experiments

TABLE I

Incorporation in vivo of galactose-1-C^{14}

Male rats, 15 days old, were given galactose-1-C^{14} (0.3 mc./mmole), 250 μc. per kg. of body weight, by intraperitoneal injection. Animals were killed at the indicated time intervals, the brains and livers were excised, and the various fractions prepared according to Procedure I (see "Experimental").

Galactose-1-C^{14} distribution of incorporated radioactivity

Time after injection	Brain fractions			Liver fractions		
	Aqueous	Acetone	Neutral glycolipide	Aqueous	Acetone	Neutral glycolipide
hrs.	%	%	%	%	%	%
2	96.0	1.9	2.1	99.5	0.4	0.1
	98.2	1.5	1.3	91.5	7.7	0.8
4	90.2	3.7	6.1	96.8	3.2	0.1
	87.8	4.0	8.2	97.8	2.0	0.1
6	87.6	4.4	8.0	98.8	1.0	0.2
	83.0	4.4	12.6	98.2	1.7	0.1
8	83.3	3.6	13.1	97.0	2.9	0.1
	87.2	2.8	10.1	98.1	1.8	0.1

TABLE II

Incorporation in vivo of glucose-U-C^{14}

Experimental details are the same as those given in Table I and in the text. The methanolysis procedure is described in the "Experimental" section.

Time after injection	Glucose-U-C^{14} radioactivity incorporation				C^{14}-glycolipide methanolysis*
	Brain fraction				
	Aqueous	Acetone	Chloroform-methanol	Neutral glycolipide	
hrs.	%	%	%	%	
2	75.6	13.8	10.6	2.1	2.5
4	76.5	13.2	10.3	4.4	7.1
	75.8	10.3	13.9	4.9	5.9
8	46.3	19.5	34.2	7.6	6.2
	43.3	24.0	32.7	4.9	2.0

* Ratio equals counts per minute of aqueous phase divided by counts per minute of organic phase. Random distribution would yield the theoretical ratio: $\frac{C_5}{C_{42}} = 0.14$.

[2] The authors wish to thank Dr. E. S. Maxwell, Dr. E. Anderson, and Dr. H. M. Kalckar for their help in preparing the UDP-galactose-1-C^{14}.

in our laboratory have shown that rats younger than 6 days do not incorporate sugars into the neutral glycolipide fraction.

Incorporation in Vivo of Galactose-1-C¹⁴ into Glycolipides of Subcellular Particles of Brain Tissue—Young male rats were given galactose-1-C¹⁴ intraperitoneally, killed after 8 hours, and the brain tissue homogenized in sucrose solution and separated into fractions by differential centrifugation after the manner of Brody and Bain (21). The neutral glycolipide fraction was prepared from each fraction by Procedure II. It may be seen that half of the C¹⁴-activity is present in the microsomal fraction (R_4), the mitochondrial fraction (R_3) accounts for about 4 to 15 per cent, the nuclear fraction (R_2) makes up about 11 to 16 per cent, the R_1 or cellular debris fraction contains 19 to 29 per cent of the radioactivity (Table III). It will be shown later in this paper that the microsomal fraction is the most active, *in vitro*, for catalyzing the incorporation of the sugar into the glycolipide.

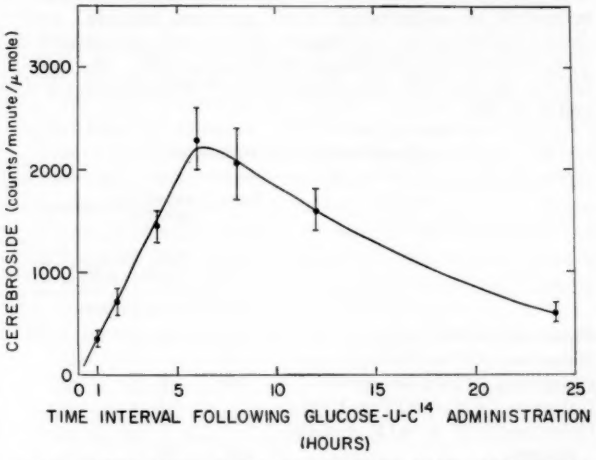


FIG. 1. Time course of the incorporation *in vivo* of glucose-U-C¹⁴ into brain tissue neutral glycolipides. In general, the experimental details are the same as those described in Tables I and II. Each point represents the average of data from at least three 14-day-old rats. The range of the cerebroside specific activity is indicated by the line through each point.

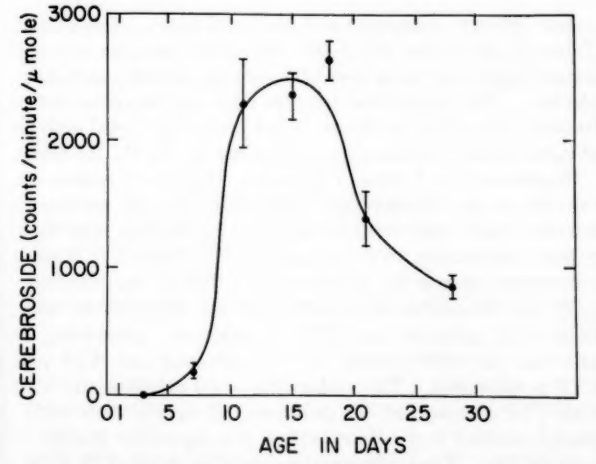


FIG. 2. Effect of age upon the incorporation of galactose-1-C¹⁴ into brain tissue neutral glycolipides. In general, the experimental details are the same as those described in Tables I and II and Fig. 1. The rats received an injection of an aqueous solution of galactose-1-C¹⁴ and were killed 8 hours later.

TABLE III

Incorporation in vivo of galactose-1-C¹⁴ radioactivity into subcellular particles of rat brain

The experimental details are the same as those described in Table I. The 15-day-old mice were killed 8 hours after the injection of hexose. The brain tissue was homogenized and separated into fractions by the procedure of Brody and Bain (21). Each experimental point represents the average of three 15-day-old male rats.

Brain particulate fraction	Galactose-1-C¹⁴ radioactivity incorporated		C¹⁴ Glycolipide methanolysis*
	Experiment 1	Experiment 2	Experiment 2
	%	%	
R_1, cell debris, nuclei and unbroken cells	19.9	29.4	4.8
R_2, nuclei	11.3	16.0	5.6
R_3, mitochondria	15.8	4.3	11.0
R_4, microsomes	53.1	50.3	2.7

* Ratio equals counts per minute of aqueous phase divided by counts per minute of organic phase. Random distribution would yield the theoretical ratio, $\frac{C_6}{C_{42}} = 0.14$.

Methanolysis of Glycolipide—The glycolipide fraction was refluxed with a chloroform-methanol-hydrochloric acid mixture. The products of the reaction were partitioned between chloroform and methanol-water. If the C¹⁴ from the hexose were metabolized and incorporated in a random manner into the glycolipide, the anticipated ratio of the radioactivity in the aqueous phase to that in the organic phase would be

$$\frac{C_6(\text{hexose fragment})}{C_{42}(\text{ceramide fragment})} = 0.14.$$

A ratio larger than 0.14 indicates preferential incorporation of the labeled carbon into the hexose moiety. The data presented in Tables II and III show that the ratio obtained for both glucose-U-C¹⁴ and galactose-1-C¹⁴ exceed the theoretical (for random distribution) ratio by a factor in excess of 20-fold, suggesting that a pathway exists for a rather direct incorporation of hexose into the neutral glycolipides which probably does not involve degradation of the sugar.

Incorporation of Glucose-U-C¹⁴ and Galactose-1-C¹⁴ into Rat Brain Neutral Glycolipides in Vitro—Glucose-U-C¹⁴ and galactose-1-C¹⁴ were incubated with a cell-free preparation of young male rat brain tissue supplemented with ATP and magnesium ions. The reaction mixture contained 5.5×10^{-3} M ATP, 5.5×10^{-3} M $MgCl_2$, 5.5×10^{-3} M uridine, 5.5×10^{-2} M nicotinamide, and 5.5×10^{-2} M potassium bicarbonate. The tissue homogenate added to each reaction mixture represented 1200 mg. of brain tissue from 19-day-old rats. Glucose-U-C¹⁴ (8.3×10^{-4} M; 0.33 mc./mmole) and galactose-1-C¹⁴ (1.1×10^{-3} M; 0.25 mc./mmole) were added as indicated. The incubation time was 2 hours at 37° under 5 per cent CO_2, N_2 and O_2 as indicated. The neutral glycolipides were isolated by Procedure I

(See "Experimental" section). Under aerobic conditions (95 per cent O_2-5 per cent CO_2) the glucose-U-C^{14} yielded 10 c.p.m. per μmole of glycolipide-galactose, and the galactose-1-C^{14} yielded 13 c.p.m. Under aerobic conditions (95 per cent N_2-5 per cent CO_2) the glucose-U-C^{14} yielded 57 c.p.m. per μmole of glycolipide-galactose, and the galactose-1-C^{14} yielded 237 c.p.m. Little incorporation of either hexose into the neutral glycolipide fraction occurred under aerobic conditions. Under anaerobic conditions the incorporation of glucose-U-C^{14} increased 5-fold and that of galactose-1-C^{14} increased some 18-fold over the aerobic level.

Effect of Heat on Hexose Incorporation—The brain control preparation, stored in ice until used, catalyzed the incorporation of both glucose-U-C^{14} and galactose-1-C^{14} into the cerebroside fraction. Galactose was incorporated to a greater extent than glucose. When the brain enzyme system was heated to 50° for 2 minutes, the incorporation of glucose was almost totally suppressed, whereas galactose incorporation was depressed only 22 per cent. In general, the experimental details are described in the preceding paragraph. The reaction mixture was incubated under anaerobic conditions (95 per cent N-5 per cent CO_2) at 37° for 3 hours. Homogenates were prepared from the brains of rats 13 days old. (*a*) One-half of the homogenate was kept chilled in ice until used. The glucose-U-C^{14} yielded 147 c.p.m., and the galactose-1-C^{14} yielded 450. (*b*) One-half of the homogenate was rapidly heated and maintained at 50° for 2 minutes then chilled in ice until used. The glucose-U-C^{14} yielded 22 c.p.m., and the galactose-1-C^{14} yielded 350. These data suggest that the pathway by which galactose can be incorporated into the cerebroside fraction is different from the glucose pathway, and further, it may be that the glucose must be converted to galactose for incorporation. The conversion of glucose to galactose may be catalyzed by the heat-sensitive enzyme UDP-galactose-4-epimerase (20, 22).

Subcellular Particle Localization of Enzyme System Incorporating Galactose into Neutral Glycolipides—The subcellular particles of brain tissue were prepared by the procedure of Brody and Bain (21). Each fraction and a recombination of all fractions were incubated with galactose-1-C^{14}, ATP, and magnesium ions. The glycolipide fraction was isolated by Procedure II and partitioned between chloroform and methanol-water. The data in Table IV show that all of the subcellular particles prepared incorporated some galactose-1-C^{14} into the neutral glycolipide fraction. The microsomal fraction (R_4) was the most active fraction accounting for about 50 per cent of the total activity obtained upon recombination of fractions R_1, R_2, R_3, R_4, and S.

TABLE IV

Distribution of enzyme activity in subcellular particles

The experimental details are similar to those described in "Results." The neutral glycolipide portion was partitioned five times between chloroform and water-methanol. The interphase precipitate was retained with the organic layer.

Subcellular particulate fraction*	Galactose-1-C^{14} incorporated	
	Chloroform phase	Aqueous phase
	c.p.m.	*c.p.m.*
R_1, cell debris fraction	24	4
R_2, nuclei fraction	31	2
R_3, mitochondria fraction	23	2
R_4, microsome fraction	86	3
S, supernatant fraction	29	2
(Total)	(193)	
R_1, R_2, R_3, R_4, S	166	3

* Prepared by the procedure of Brody and Bain (21).

TABLE V

Incorporation of uridine diphosphogalactose-1-C^{14} into rat brain galactolipide

The reaction mixtures all contained 7.7×10^{-2} M potassium phosphate, 7.7×10^{-3} M $MgCl_2$, and microsomal preparations (R_4) equivalent to 1 gm. wet weight of rat whole-brain tissue. The total volume was 1.3 ml., pH 8. The incubation period was 3 hours at 37° under 95 per cent nitrogen-5 per cent carbon dioxide. Other additions were made as indicated to give the following concentrations: 7.7×10^{-3} M ATP, 1.5×10^{-3} M UTP, 1.5×10^{-3} M UDP-glucose, 6.1×10^{-4} M galactose-1-C^{14} (0.25 mc. per mmole), 7.7×10^{-4} M galactose(-1-C^{14})-1-phosphate (0.25 mc. per mmole), 7.7×10^{-4} M UDP galactose-1-C^{14} (0.25 mc. per mmole). The neutral glycolipides were isolated from the reaction mixture by Procedure II. Experiment 3 demonstrates the magnitude of radioactivity in the zero-time controls. Immediately following the last reagent addition, an unincubated aliquot was removed and added to 20 volumes of CM. The remaining reaction mixture was incubated at 37° before an aliquot was added to CM.

	Galactose radioactivity incorporated			
	Experiment 1	Experiment 2	Experiment 3	
	Incubated	Incubated	Unincubated	Incubated
	c.p.m.	*c.p.m.*	*c.p.m.*	*c.p.m.*
Galactose-1-C^{14} + ATP	21	9	9	16
Galactose-1-C^{14} + UDP-glucose	18			
Galactose(-1-C^{14})-1-phosphate	15	5		
Galactose-1-C^{14} + ATP + UTP		4		
Galactose-1-C^{14} + ATP + UDP-glucose	205	84		
Galactose(-1-C^{14}-)1-phosphate + UDP-glucose	183			
Galactose-1-C^{14} + ATP + UDP-glucose (heat inactivated control)	3			
UDP-Galactose-1-C^{14}		106	4	77

Requirement for Uridine Nucleotides—The role of uridine nucleotides in the incorporation of galactose into the cerebroside fraction of rat brain tissue is illustrated by the data from three typical experiments which are presented in Table V. Washed microsomes catalyze the incorporation of little, if any, galactose-1-C^{14} into the neutral glycolipides with the supplemental additions of (*a*) galactose and ATP, (*b*) galactose-1-phosphate, (*c*) galactose and UDP-glucose, and (*d*) galactose and ATP plus UTP as substrates. The combination of (*a*) galactose and ATP with UDP-glucose and (*b*) galactose-1-phosphate with UDP-glucose resulted in the incorporation of a significant amount of radioactivity. These combinations could be replaced by UDP-galactose-1-C^{14}. The appropriate heat-inactivated controls showed no incorporation.

The results of the methanolysis of the neutral glycolipide fractions obtained from typical experiments, *i.e.* glucose-U-C^{14},

galactose-1-C^{14}, and UDP-galactose-1-C^{14} used as the C^{14} donor, are summarized by the data presented in Table VI.

Identification of Radioactive Glycolipide—Properties of the material obtained by the extraction procedures and the mixed-bed column (Nalcite-SBR, and -HCR, and Florisil) indicate that the radioactivity was incorporated into the neutral glycolipide fraction (17). This fraction has been shown, by Radin (17) and in the experiments reported in this paper, to contain a sugar by either the anthrone (17) or carbazole (23) color tests. Glycolipide fractions, carefully removed from planchets with CM, were taken to dryness and suspended in chloroform and their infrared spectra determined. Major peaks were observed which corresponded to that of an authentic cerebroside sample (24). In addition, strong absorption at 5.76 μ (probably a result of an ester carbonyl group) was seen. After the methanolysis procedure, the chloroform-soluble material was treated as described by Brady and Burton (25) for the purification of sphingosine. The resultant heptane-soluble fraction gave an infrared spectrum which was identical with that of sphingosine base. The aqueous fraction, obtained by methanolysis, was recovered from the counting planchet with pyridine. Subsequent crystallization from ethanol yielded a product which gave an infrared spectrum in pyridine similar to that of methyl galactoside but differing from that of methyl glucoside by a moderate absorption peak at 8.79 μ (26). The major portion of the radioactivity was associated with this water soluble fraction.

The neutral glycolipide fraction prepared from rat brain tissue after the injection of galactose-1-C^{14} *in vivo*, was isolated by the use of the mixed-bed column. The neutral lipide fraction was subsequently chromatographed on silicic acid using a procedure similar to that of Weiss (10). Elution from the column was performed by changing solvents rather than by the gradient elution procedure described by Weiss. Typical results are presented in Fig. 3. A radioactive lipide was eluted with chloroform and was shown to be a glycolipide. Elution with chloroform-methanol, 10:1, gave a second radioactive peak which gave a positive anthrone sugar test. Purified beef brain cerebroside (phrenosin) labeled with tritium was eluted from the silicic acid column with chloroform-methanol 10:1. It is therefore probable that the second radioactive glycolipide eluted from the column is phrenosin. The first glycolipide is probably similar to the compound that was mentioned by Weiss (10), *i.e.* N-lignoceryl-O^1-galactosyl sphingosine. That portion of Fig. 3 labeled "*in vitro* experiment" represents the results of chromatographing the radioactive glycolipide fraction obtained from an incubation experiment *in vitro* on a silicic acid column. Only one radioactive peak was detected and the position of this peak corresponded to that of phrenosin.

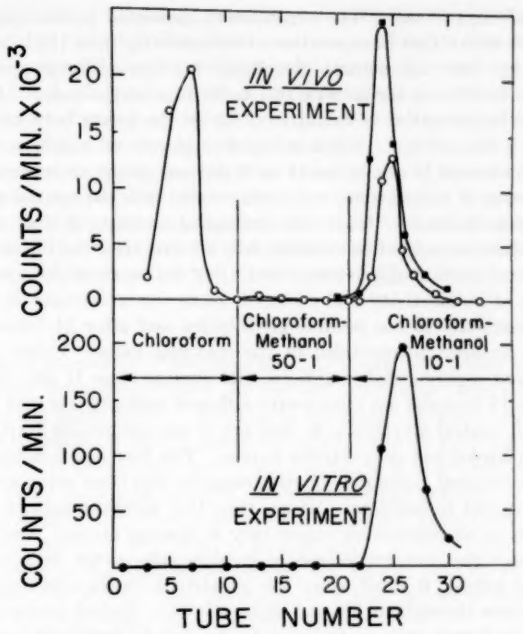


FIG. 3. Silicic acid chromatography of brain tissue glycolipides. A slurry of silicic acid in chloroform-methanol, 2:1, was used to prepare a column of silicic acid 90 × 13 mm. The column was washed with chloroform (about 50 ml.) until the silicic acid was translucent. The neutral glycolipid (Procedure II) was placed on the top of the silicic acid in 0.2 ml. of CM and washed into the column with 0.4 ml. of CM. The glycolipides were eluted from the column with (*a*) chloroform, 100 ml., (*b*) chloroform-methanol, 50:1, 100 ml., and (*c*) chloroform-methanol, 10:1, 100 ml. An aliquot from each tube was analyzed for hexose by the anthrone method. Radioactivity in each tube is indicated as follows: ■, purified cerebroside (phrenosin) labeled with tritium; ○, neutral glycolipides from rat brain tissue after an intraperitoneal injection of galactose-1-C^{14}, and ● neutral glycolipide from the incubation *in vitro* of a rat brain microsomal preparation with UDP-galactose-1-C^{14}.

TABLE VI

C^{14} Galactolipide methanolysis

The galactolipide fractions were subjected to methanolysis as described in "Experimental."

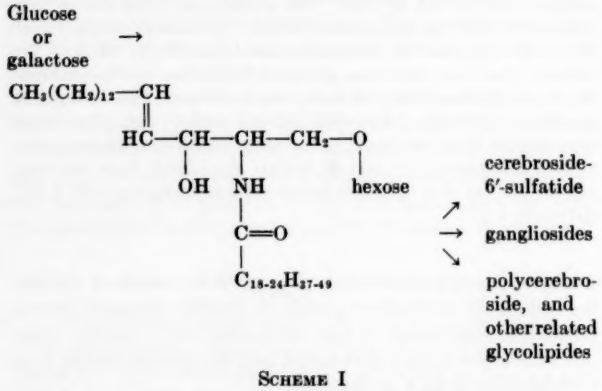
Substrate incorporated into galactolipide	Aqueous phase	Organic phase	Ratio = c.p.m. aqueous* / c.p.m. organic
	c.p.m.	*c.p.m.*	
Glucose-U-C^{14}	81	13	6.2
Galactose-1-C^{14}	123	37	3.3
Galactose-1-C^{14}	30	16	2.0
UDP-Galactose-1-C^{14}	41	10	4.1
UDP-Galactose-1-C^{14}	39	8	4.9

* Random incorporation would yield a theoretical ratio of $\frac{C_6}{C_{42}} = 0.14$.

DISCUSSION

The chemical composition of the developing brain undergoes very rapid and extensive changes during the first few weeks *post partum* in the rat and mouse (27–29). No myelination is discernible by histological techniques before the age of about 8 days in the mouse and is probably complete by the age of 50 days (27). Cerebrosides are a constituent of the myelin sheath and begin to accumulate in brain tissue at about the 8th day (27). It is not surprising that the incorporation of galactose and glucose into the glycolipide fraction of brain *in vivo* correlates both with the histological demonstration of myelination occurring after the 8th day and with the data of Folch (27) showing that cerebrosides are present in very low amounts before 8 days of age and accumulate largely between the 8th and,

22nd days of age. The experiments, presented in this paper, have shown that incorporation of radioactivity from the labeled hexose into the neutral glycolipide fraction of brain occurs mainly between the interval of 7 to 28 days *post partum*. Maximal incorporation is found to occur in the group between 11 to 18 days of age. When radioactive glucose or galactose was administered to rats in the 11 to 18 day age group, an increasing amount of radioactivity was incorporated into the neutral glycolipide fraction of brain with increasing amounts of time, and maximal incorporation occurred 6 to 8 hours after the injection. It is of considerable interest that after the maximal incorporation of radioactivity had occurred, there was a decrease in the radioactivity in the neutral glycolipides and after 24 hours it had dropped to one-third of the maximal value. Under the present experimental conditions, rats younger than 11 and older than 18 days did not incorporate sufficient radioactivity into the brain neutral glycolipide to indicate if the cerebroside fraction thus labeled lost radioactivity *in vivo*. This loss in radioactivity in the neutral glycolipide fraction suggests that these compounds are indeed metabolized. If so, then this turnover appears to occur to an appreciable extent only in this age group. Metabolism of the cerebroside fraction in older rats occurs to a very small extent, if at all, since the quantity of brain cerebrosides increases throughout life and since only very limited incorporation of hexose into neutral glycolipides can be demonstrated in older animals. Another possibility is that "turnover" of the cerebroside fraction itself is very limited but that in addition to the synthesis of neutral glycolipides by young animals, the formation of other glycolipids, *e.g.* gangliosides and cerebroside sulfate, occurs via neutral glycolipid precursors.



SCHEME I

Thus the neutral glycolipide fraction would become labeled from the radioactive hexose and after about 8 hours the labeled hexose will have been metabolized and the circulating sugar will be unlabeled (or very low), therefore additional neutral glycolipides synthesized will be essentially unlabeled. Part of the cerebrosides in the brain could be constantly removed by being incorporated into more complex structures and would be replaced by glycolipides formed from unlabeled hexose. The specific activity of the cerebral neutral glycolipide would decrease.

The investigations of Leloir (30, 31), and Kalckar *et al.* (32, 33) have shown that the uridine nucleotides are frequently involved in glycosyl transfer reactions, *e.g.* UDP-glucose donates glucose to fructose to form sucrose, to fructose-6-phosphate to form sucrose phosphate, and to a primer to form glycogen (34–36). Uridine is also involved in the interconversion of glucose and galactose (20, 30). It is therefore proposed that the following series of equations may describe the step-wise reaction sequence for the incorporation of glucose and galactose into the neutral glycolipide.

Glucose Pathway

$$\text{Glucose} + \text{ATP} \rightleftharpoons \text{glucose-6-phosphate} + \text{ADP} \quad (1)$$

$$\text{Glucose-6-phosphate} \rightleftharpoons \text{glucose-1-phosphate} \quad (2)$$

$$\text{Glucose-1-phosphate} + \text{UTP} \rightleftharpoons \text{UDP-glucose} + \text{pyrophosphate} \quad (3)$$

$$\text{UDP-glucose} \rightleftharpoons \text{UDP-galactose} \quad (4)$$

$$\text{UDP-galactose} + \text{acceptor} \rightleftharpoons \text{neutral glycolipide} + \text{UDP} \quad (5)$$

Galactose Pathway

$$\text{Galactose} + \text{ATP} \rightleftharpoons \text{galactose-1-phosphate} + \text{ADP} \quad (6)$$

$$\text{Galactose-1-phosphate} + \text{UDP-glucose} \rightleftharpoons \text{UDP-galactose} + \text{glucose-1-phosphate} \quad (7)$$

$$\text{UDP-galactose} + \text{acceptor} \rightleftharpoons \text{neutral glycolipide} + \text{UDP} \quad (5)$$

The evidence in support of these reactions may be summarized as follows: (*a*) Both glucose and galactose will give rise to neutral glycolipides *in vivo*, and both hexoses will be incorporated if the enzyme system from brain is supplemented with ATP and if the incubation is carried out under anaerobic conditions *in vitro*. The glycolipide synthesizing enzyme system is relatively slow in comparison with many other pathways of hexose utilization and competes very poorly with these other pathways. (*b*) Heating the enzyme system to 50° for 2 minutes leaves the system incapable of the incorporation of glucose; galactose, however, may still be incorporated into the neutral glycolipide. It is known that UDP-galactose-4-epimerase is sensitive to heat and may be inactivated under these conditions (20). According to the proposed sequence (Equations 1 to 5), if the epimerase (Equation 4) were inactivated and if UDP-galactose is the more effective donor to the lipide, then glucose would not be incorporated into the neutral glycolipide. The possibility that one of the enzymes required before the epimerase (*i.e.* Equations 1 to 3) was inactivated by the heat treatment has not been excluded. (*c*) It has been shown by the data in this paper that galactose-1-phosphate can be used in place of galactose plus ATP. (*d*) Washed microsomal preparations of young rat brain often failed to incorporate galactose plus ATP or galactose-1-phosphate unless the reaction mixture was supplemented with UDP-glucose. UTP would not replace the UDP-glucose requirement. However, it was found that UDP-galactose could replace the mixture of galactose plus ATP supplemented with UDP-glucose Since UDP-galactose is apparently the immediate galactosyl donor to the lipide acceptor, it is obvious that galactose-1-phosphate is incorporated into the nucleotide form via the reaction represented by Equation 7 and not by a reaction analogous to the UTP-glucose-1-phosphate transfer (Equation 3). Hexokinase (Equation 1), phosphoglucomutase (Equation 2), UDP-glucose pyrophosphorylase (Equation 3), and UDP-galactose-4-epimerase (Equation 4) have all been demonstrated in brain tissue (19, 37, 38). Galactose utilization has been shown to occur in brain homogenates (39). Leloir has reported the presence of galactokinase in brain tissue (31). Recently,

Kurahashi[3] has obtained evidence for the presence of galactose-1-phosphate uridyl transferase (Equation 7) activity in brain tissue. The data presented in the present experiments support the reaction indicated by Equation 5.

The radioactivity has been found to be incorporated mainly into the hexose moiety of the neutral glycolipide fraction. This result is to be expected in the experiments studied *in vitro* if the enzyme system operates via the proposed series of reactions. It is interesting to note that preferential incorporation is also observed in the studies conducted *in vivo*. These results must mean that either the lipide acceptor, which is presumed to be either sphingosine base or *N*-acyl sphingosine (ceramide), is already present in sufficient quantity or that the formation of the lipide acceptor occurs by processes reasonably removed, in time, from the immediate metabolic products of the hexoses. The C^{14}-hexoses evidently must have sufficient opportunity to be incorporated *per se* before metabolism and random distribution can occur. The data of Moser and Karnovsky (13) clearly indicate that a direct incorporation of hexose does occur.

The infrared spectrum of the lipide fraction isolated from the mixed-bed resin column and partitioned between water and the organic solvent was quite similar to that of cerebroside isolated and purified from beef spinal cord lipide. An additional absorption maximum was observed at 5.76 μ, probably due to an ester carbonyl group, which may indicate that the cerebroside fraction contains a neutral lipid, perhaps a simple ester. The radioactivity was found to be in the water-soluble product obtained by methanolysis of the cerebroside fraction. This product is probably methyl galactoside. It is interesting to observe that the experiments *in vivo* yielded a neutral glycolipide fraction which produced two radioactive fractions when chromatographed on silicic acid. One of these peaks corresponded to that of a purified phrenosin standard and was chromatographically similar to the radioactive peak obtained from the experiments *in vitro*. From the data reported by Weiss (10), by Radin *et al.* (11), and by comparison with our cerebroside standard, it is probable that these fractions contain *N*-cerebronyl-O^1-galactosyl sphinogsine, whereas the first radioactive glycolipide eluted from the silicic acid in the experiment *in vivo* is probably comparable with the glycolipide reported by Weiss (10), *i.e.* *N*-lignoceryl-O^1-galactosyl sphingosine. It was of considerable interest to learn that the cerebroside containing an α-hydroxy fatty acid was preferentially formed *in vitro*. This finding may prove to be significant for the elucidation of the detailed sequence of reactions for the biosynthesis of glycosphingolipides.

SUMMARY

Both glucose-U-C^{14} and galactose-1-C^{14} have been shown to be readily incorporated into the neutral glycolipide fraction of rat brain tissue *in vivo*. The rate of incorporation of the hexoses was correlated with the ages of the animals and maximal specific activity of the glycolipide fraction was found in rats which were 10 to 20 days *post partum*. Maximal specific activity of the neutral glycolipides was observed 8 hours after a single intraperitoneal injection of glucose or of galactose. 24 hours after the injection of the radioactive hexose, the specific activity of the glycolipide fraction had decreased to about one-third that of the maximal value observed (at 8 hours).

The experiments conducted *in vitro* showed that the microsomal fraction of young rat brain contains the enzymes necessary for the incorporation of glucose and galactose into neutral glycolipides. It was found that uridine diphosphogalactose was the primary hexose donor to a lipide acceptor endogenous to the microsomal fraction. Chromatographic and infrared spectral studies indicate that the radioactive glycolipide isolated from the experiments *in vitro* is a cerebroside, probably *N*-cerebronyl-O^1-galactosyl sphingosine.

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Purification and Identification of Brain Phospholipides Associated with Thromboplastic Activity*

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(Received for publication, May 12, 1958)

Early work has demonstrated the blood-coagulating activity of phospholipides from brain tissue and platelets (1–4). More recent studies have indicated that platelets can be replaced in most coagulation tests by phospholipides prepared from brain or platelets (5–9). While continuing previous work (10) on the isolation of the active thromboplastic phospholipides from brain tissue the authors noted that the specific activity of the purified fractions never exceeded the specific activity of the original preparation but actually seemed to diminish upon refractionation. Similar observations were made by Rapport (11) and by Biggs and Bidwell (12). Furthermore, Rapport (11) observed that a combination of brain cephalin fractions with lecithin in nonpolar organic solvents yielded preparations with greater thromboplastic activity than the cephalin tested alone. Lecithin alone exhibited no activity.

In order to examine further the question of the thromboplastic activity of brain tissue, the phospholipides from beef brain were fractionated by various means, and the action of each fraction was tested both alone and after combination with lecithin. In this paper results are described which indicate that a phospholipide fraction can be obtained from brain tissue which by itself has little or no thromboplastic activity but which upon combination with lecithin yields a phospholipide with very high thromboplastic potency.

EXPERIMENTAL

Preparation of Phospholipide Fractions

Folch's Fraction III (primarily phosphatidyl serine) and Fraction V (primarily phosphatidyl ethanolamine) were prepared from beef brain tissue according to the procedure of Folch (13). An ethanol extract from acetone-dried beef brain tissue (13) was used for the preparation of lecithin by the method of Pangborn (14).

Countercurrent Distribution Fractionation—Both the lecithin and the Fraction III preparations were further purified by countercurrent distribution. An all glass Craig 100 cell countercurrent distribution apparatus was used. The solvent system consisted of carbon tetrachloride, methanol, and water in the ratio of 62:35:4. The phospholipide to be fractionated was dissolved in 10 ml. of the lower phase and equilibrated with an equal volume of the upper phase. This mixture was placed in tube 0. In this instrument the bottom phase was stationary whereas the top phase traveled along the train. Amounts of phospholipide varying between 0.3 and 2.0 gm. were fractionated in individual runs.

Characterization of Phospholipides

The various phospholipide fractions obtained by countercurrent distribution were identified and characterized by the following means: (*a*) paper chromatography of the unhydrolyzed phospholipides (by the method of Marinetti *et al.* (15)) on silicic acid-impregnated paper, using ascending technique with the solvent mixture diisobutyl ketone-acetic acid-water, 40:25:5 by volume at 23° or 40:20:3 at 0°; (*b*) spot test for unhydrolyzed phospholipides with Rhodamine 6 G (16); (*c*) chromatography of nitrogenous phospholipide bases by the method of Levine and Chargaff (17); (*d*) ninhydrin spot test for α-amino groups (16); and (*e*) phosphomolybdic acid spot test for choline (18).

Determination of Thromboplastic Activity

The thromboplastic activity of the various phospholipide fractions was determined in two ways.

1. Acceleration of the clotting time of recalcified plasma—0.2 ml. of oxalated rabbit plasma was transferred to a small test tube, and 0.1 ml. of the phospholipide suspension was added. This mixture was incubated at 37° for 5 minutes, after which 0.2 ml. of $CaCl_2$ (0.025 M) was blown forcibly into the tube by means of a pipette. A stop watch was started at this moment, and the clotting time was determined at 37° by the tilt method. The phospholipides had been thoroughly emulsified in a saline solution of pH 7.2.

2. Formation of a prothrombin conversion factor—A modification of the thromboplastin generation test of Biggs and Douglas (19) was used.

Materials—The serum eluate preparation was obtained from aged rabbit serum as previously described (9). The proconvertin activity of this preparation, determined according to the method of Owren and Aas (20), was found to be about 400 units per mg. One unit of proconvertin was arbitrarily defined as that amount of preparation which will give a clotting time equal to that of 0.1 ml. of 1:50 dilution of normal rabbit plasma as assayed by the above method. The serum eluate preparation employed may not constitute a single entity and may also contain, in addition to proconvertin, plasma thromboplastin component. A solution containing 0.05 mg. per ml. in saline at pH 7.2 was used in the assay. The $BaSO_4$-treated plasma (oxalated rabbit plasma) was stirred for 20 minutes with $BaSO_4$ (Baker's; 100 mg. per ml.), centrifuged, and decanted. This plasma was diluted 1:5 with saline. A solution of 0.025 M

* The term "thromboplastic activity" refers to that of an agent which cannot by itself directly activate prothrombin but must first react with certain other clotting factors in the presence of calcium ions to form a prothrombin conversion factor.

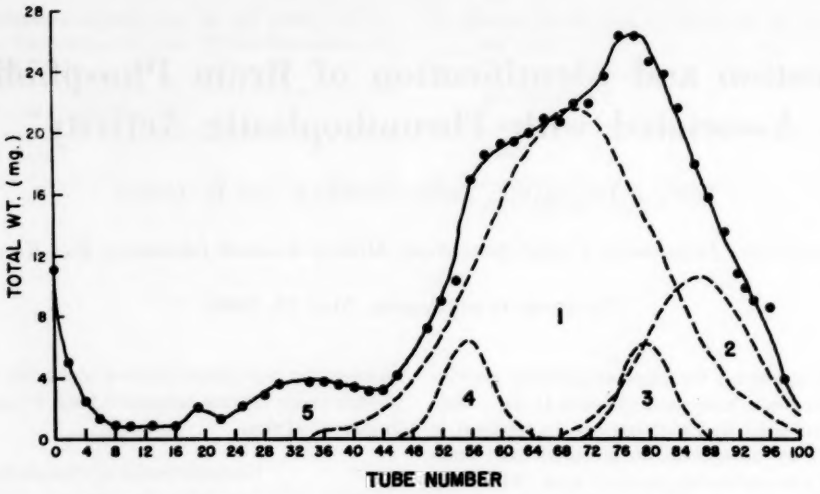

FIG. 1. Countercurrent distribution of Folch Fraction III. ●——●, represents the total dry weight. Area under ----, indicates the individual components: 1, phosphatidyl serine; 2 and 3, inositol phosphatides; 4, unidentified; and 5, phosphatidyl ethanolamine and lyso-phosphatidyl ethanolamine.

$CaCl_2$ in water was used. The phospholipide to be assayed was thoroughly emulsified in saline of pH 7.2. The concentration of the phospholipide emulsion was 0.025 mg. per ml.

Procedure—A reaction mixture was prepared consisting of 0.5 ml. of each of the following reactants: serum eluate, $BaSO_4$-treated plasma, phospholipide emulsions, and $CaCl_2$. At various time intervals of incubation at 26°, 0.1 ml. of the reaction mixture and 0.1 ml. of $CaCl_2$ solution were simultaneously added to 0.1 ml. of normal oxalated rabbit plasma, and the clotting times (as obtained by the tilt method) were recorded.

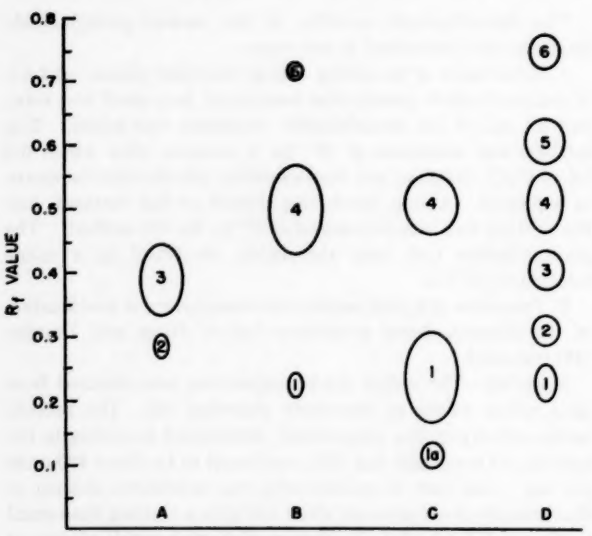

FIG. 2. Representation of paper chromatography of phospholipide fractions from countercurrent distribution.

RESULTS

Fractionation and Characterization—The results of the countercurrent distribution of Folch's Fraction III are shown in Fig. 1. The major component, 1, indicated by the broken line, represents phosphatidyl serine. However, there are several other contaminating substances present. Fig. 2 is a reproduction of the paper chromatographic analysis of the material isolated from the various segments indicated by the curves from Figs. 1 and 3. The phospholipide spots were developed by staining with Rhodamine 6 G. The spots were further characterized by means of the ninhydrin and the phosphomolybdic acid tests. Spot 4 has the same R_F value as that of phosphatidyl serine in the standard mixture. It gave a positive ninhydrin but a negative choline test. Spots 1 and 1a, on the other hand, gave a negative test for choline and a negative ninhydrin test. Since Spot 1 has the same R_F value as the inositol phosphatide of the standard mixture, it is presumed to be inositol phosphatide. The slower moving component, 1a, is believed to be another inositol phosphatide. Spot 6 has an R_F value identical to that of diglycerol phosphate in the standard mixture and gave neither a positive ninhydrin nor positive choline test. In addition to phosphatidyl serine and an unidentified phospholipide, the slower moving component from the countercurrent distribution obtained from tubes 20 to 45 (Fig. 1) was shown by paper chromatography to consist of phosphatidyl ethanolamine and possibly lyso-phosphatidyl ethanolamine. Paper chromatography of the nitrogen-containing bases, obtained by hydrolysis of the phospholipide, also showed that the NH_2 group detected in the material present in tubes 60 to 90 (Fig. 1) was entirely due to serine; there was no ethanolamine present. However, the NH_2 groups detected in the material obtained from tubes 20 to 45 (Fig. 1) were primarily due to ethanolamine. From these data it is possible to construct a series of Gaussian distribution curves indicating the relative inhomogeneity of Fraction

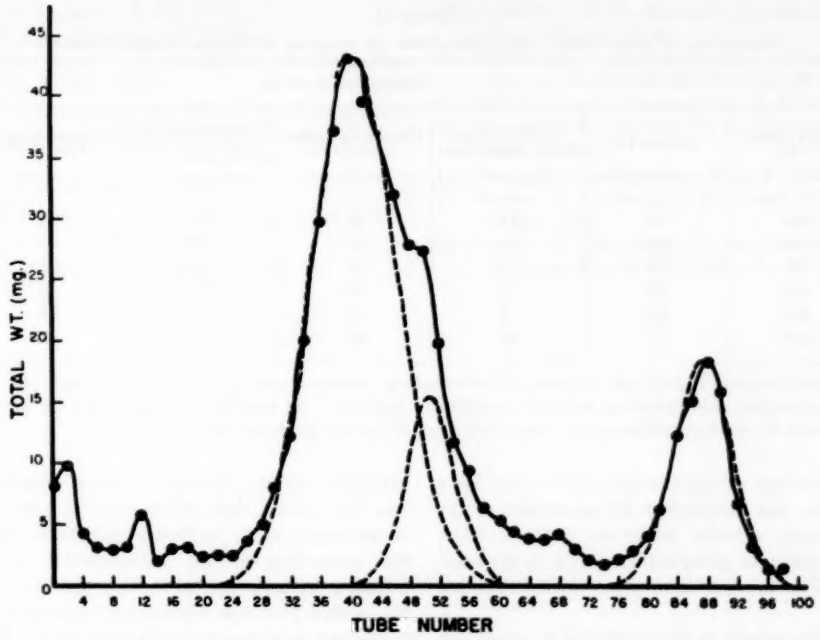

FIG. 3. Countercurrent distribution of lecithin prepared by the method of Pangborn. ●——●, represents the total dry weight. Area under ----, indicates individual components. Lecithin is in tube 40, sphingomyelin, in tube 52, and an unidentified component is in tube 88.

III and the degree of separation achieved by countercurrent distribution. The area under *Curve 1* of Fig. 1 is phosphatidyl serine, and *Curves 2* and *3* indicate the two inositol phosphatides. *Curve 4* is believed to be diglycerol phosphate, although it has not as yet been characterized, and *Curve 5* contains phosphatidyl ethanolamine and possibly lyso-phosphatidyl ethanolamine.

The results of the countercurrent distribution of lecithin prepared by Pangborn's method are shown in Fig. 3. There are at least three substances present in this preparation. The major component was characterized by paper chromatography as lecithin. Spot 3 (Fig. 2) has the same R_F value as lecithin in the standard mixture. It gave a negative ninhydrin and a positive choline test. The minor spot (number 2) has the same R_F value as sphingomyelin in the standard mixture, and it too gave a negative ninhydrin and a positive choline test. Material obtained from tubes 50 to 60 (Fig. 1), on the other hand, contained a much greater proportion of sphingomyelin, which indicates that sphingomyelin has a slightly higher partition coefficient than lecithin. Paper chromatography of the hydrolysis products of both of these fractions indicated that the positive phosphomolybdic acid test was actually due to choline. The faster moving component with a peak at tube 88 has not been identified. It elicited no thromboplastic activity either alone or in combination with lecithin.

Thromboplastic Activity—The effect of the phospholipide fractions on the recalcification time of normal oxalated rabbit plasma is shown in Table I. It can be seen that lecithin has no effect on the recalcification time whereas Folch's Fraction III seems to produce a very slight reduction at high concentrations. However, if Fraction III and lecithin are first mixed in the ratio of 1:2 in chloroform solution, the material, after evaporation of the chloroform under nitrogen, exhibits a very potent accelerating effect on the clotting time of recalcified plasma. Even at levels of 10^{-5} μg. the clot-accelerating effect of the mixture was still detectable. After countercurrent distribution of Fraction III, the phosphatidyl serine fraction showed somewhat less activity than the Fraction III preparation. This purified phosphatidyl serine fraction, when mixed with purified lecithin, exhibited great thromboplastic activity (Table I).

TABLE I

*Influence of phospholipide preparations on recalcification time of rabbit plasma**

Phospholipide	Lecithin†	Fraction III	Fraction III-lecithin‡	Phosphatidyl serine§	Phosphatidyl serine-lecithin‡
μg.	sec.	sec.	sec.	sec.	sec.
100	111	57	55	81	56
10	121	111	50	95	42
1	130	123	52	109	41
10^{-1}		142	64	141	57
10^{-2}			75		68
10^{-3}			63		83
10^{-4}			85		75
10^{-5}			99		81

* Recalcification time of normal oxalated rabbit plasma = 132 sec.

† Fraction obtained by countercurrent distribution, tubes 31 to 45 (Fig. 3).

‡ Fraction III or phosphatidyl serine dissolved in $CHCl_3$ with lecithin in the ratio of 1:2 by weight.

§ Fraction obtained by countercurrent distribution of Fraction III, tubes 60 to 75 (Fig. 1).

TABLE II

Formation of prothrombin conversion factor in presence of various phospholipides

Incubation time of reaction mixture*	Clotting time in seconds						
	Lecithin (tubes 31–45)	Fraction III	Fraction III and lecithin (tubes 31–45)	Phosphatidyl serine (tubes 60–75)	Phosphatidyl serine (tubes 60–75) and lecithin (tubes 31–45)	Phosphatidyl serine (tubes 75–90)	Phosphatidyl serine (tubes 75–90) and lecithin (tubes 31–45)
min.							
1	100	31	15	44	12	34	13
2	81	30	9	38	10	35	10
3	92	30	7	35	11	37	11
5	92	30	9	39	10	35	11
8	91	32	8	43	11	35	9
15	96	31	10	45	11	42	12

* The reaction mixture consisted of 0.5 ml. of each of the following: serum eluate preparation (0.05 mg. per ml.), $BaSO_4$-treated plasma (1:5 dilution), phospholipide (0.025 mg. per ml.), and $CaCl_2$ (0.025 M). At intervals indicated, 0.1 ml. of the reaction mixture and 0.1 ml. of $CaCl_2$ (0.025 M) were simultaneously added to 0.1 ml. of normal oxalated rabbit plasma.

In order to test whether sphingomyelin, which may be a contaminant of lecithin, was involved in the generation of the increased thromboplastic activity observed with lecithin, sphingomyelin was mixed with phosphatidyl serine in the same manner as lecithin. The mixture showed no clot-accelerating activity.

Table II illustrates the results of the prothrombin conversion factor generation test which involved these same phospholipide fractions. Again it can be noted that lecithin has no effect on the generation of prothrombin-converting activity. Fraction III alone apparently generated very little prothrombin conversion factor. However, when a mixture of Fraction III and lecithin was employed in the incubation mixture, the generation of a prothrombin conversion factor was greatly enhanced and essentially complete within 2 minutes. Both phosphatidyl serine fractions obtained from tubes 60 to 75 and from tubes 75 to 90 (Fig. 1) were slightly less active than Fraction III. However, when either one of these phosphatidyl serine fractions was mixed with lecithin, each of the incubation mixtures resulted in the generation of a potent prothrombin conversion factor within 1 minute (Table II).

DISCUSSION

The results of this study clearly demonstrate that combination of phosphatidyl serine and lecithin results in the formation of an active thromboplastic substance. The identification of the various fractions by paper chromatography (Fig. 2) shows that the phosphatidyl serine fraction obtained from tubes 55 to 65 (Fig. 1) contains, in addition to phosphatidyl serine, minute traces of inositol phosphatide and an unidentified phospholipide. On the other hand, the phosphatidyl serine fraction obtained from tubes 80 to 100 (Fig. 1) contained, in addition to phosphatidyl serine, a large amount of inositol phosphatide but none of the unidentified phospholipide. Both of these fractions, when assayed by the recalcification time and the prothrombin conversion factor generation test, showed activity of the same order of magnitude which indicated that the activity could not be attributed to the presence of these minute contaminants. It was also shown that sphingomyelin, which may have been a contaminant of the lecithin preparations, did not contribute to the generation of the prothrombin conversion factor. In addition, for assay purposes, only the slower moving portion of the lecithin peak was taken from the countercurrent distribution to insure a lecithin free of sphingomyelin. This evidence makes it relatively certain that the two entities responsible for the thromboplastic activity, as measured in these experiments, are lecithin and phosphatidyl serine. These results are in no way contrary to the findings of Rouser *et al.* (21) who have shown that phosphatidyl ethanolamine has thromboplastic activity. It has been our experience that thromboplastic activity is also associated with phosphatidyl ethanolamine.

Evidence that interaction may have taken place between lecithin and phosphatidyl serine when they were mixed in chloroform, and that the thromboplastic activity was not due to the solubilizing effect of one phospholipide on the other, may be deduced from the finding that no augmented thromboplastic activity could be generated when the same components were emulsified in water without being previously mixed together in chloroform solution. The nature of this interaction is still unknown. However, infrared absorption studies indicate that this interaction is not a covalent one.

SUMMARY

Phosphatidyl serine and lecithin have been isolated from beef brain tissue by means of countercurrent distribution and identified.

Neither phosphatidyl serine nor lecithin alone possesses any appreciable thromboplastic activity. When lecithin and phosphatidyl serine are dissolved together in chloroform, however, a potent thromboplastic substance results.

Acknowledgment—The authors wish to thank Dr. G. V. Marinetti of the University of Rochester School of Medicine and Dentistry for his assistance and advice.

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Studies on the Metabolism of 3,3-Dimethyl Phenylmyristic Acid, a Nonoxidizable Fatty Acid Analogue

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(Received for publication, July 9, 1958)

The small quantities of unesterified fatty acid in the blood plasma of mammals have recently been shown to be of great metabolic significance, apparently representing a lipide fraction readily available as a substrate for oxidation (1). These fatty acids are predominantly transported in plasma as a complex with serum albumin (2).

Further information regarding the metabolic significance of this transport mechanism might be obtained if it were possible to introduce a fatty acid analogue which could (*a*) compete effectively with normally occurring fatty acids for the binding sites on serum albumin, and (*b*) resist oxidative degradation or metabolism by other pathways. Such a compound, given in sufficient quantity, might arrest or sharply depress the normal rate of UFA[1] transport. If the latter is in fact the major transport form for fatty acids in the fasting state, interference with such transport might be expected to have profound effects on the metabolism of the fasting animal and possibly on the concentrations and rates of turnover of serum lipoproteins.

The compound selected for this study was 3,3-dimethyl-14-phenylmyristic acid. Recent investigations by Bergström *et al.* (3) and by Tryding and Westöö (4) have demonstrated that β-oxidation of fatty acids does not take place in the absence of hydrogen atoms on the α-carbon. Thus, 2,2-dimethyl stearic and nonadecanoic acids were almost completely excreted in the urine as 2,2-dimethyl short chain dicarboxylic acids. It was therefore anticipated that the above acid would be inert to β-oxidation; the 3,3 substitutions were selected (rather than the 2,2) so as to provide less steric hindrance for binding to serum albumin. Tryding and Westöö (5) have also shown that 2,2,17,17-tetramethyl stearic acid is inert to both β- and ω-oxidation, being excreted in the feces largely as the unchanged acid. Because of the combined requirements in the present study for tight binding to serum albumin and for relatively high solubility of the fatty acid salt, it was felt that an aromatic substitution at the ω end of the fatty acid would be a more suitable way to interfere with ω-oxidation. Stevens (6) has shown that a β-phenyl substitution interferes with β-oxidation and a similar effect was therefore anticipated for ω-oxidation.

The present paper describes the synthesis of a mixture of two isomers, 3,3-dimethyl-14-phenylmyristic acid and 3,3-dimethyl-13-phenylmyristic acid (DPMA). The binding of this fatty acid analogue to serum albumin has been measured relative to the binding of palmitic acid. After intravenous injection in rats the rate of disappearance of DPMA from plasma, excretion in urine and feces, body distribution, and extent of incorporation into several lipide fractions have been determined, as well as the effects on oxidation of palmitic acid.

[1] The abbreviations used are: UFA, unesterified fatty acid; DPMA, a mixture of 3,3-dimethyl-13-phenylmyristic acid and 3,3-dimethyl-14-phenylmyristic acid.

METHODS AND MATERIALS

A. Synthesis—3,3-Dimethyl-14 (and 13)-phenylmyristic acid was prepared by electrolytic synthesis, as described by Stallberg-Stenhagen (7), and also by Bergström *et al.* (3) and Tryding and Westöö (4, 5). The starting materials were 3,3 dimethyl glutaric acid monomethyl ester and phenyl undecanoic acid. The latter was an Eastman Kodak Company chemical; it had been prepared by a Friedel-Crafts synthesis from 10-undecenic acid, and was a mixture of 10-phenyl and 11-phenyl undecanoic acids (mostly the 10 isomer). 3,3-Dimethyl glutaric monomethyl ester was prepared by refluxing 3,3-dimethyl glutaric anhydride (Aldrich Chemical Company) with methanol; the infrared spectrum after refluxing for 60 minutes showed no anhydride peak, with a split peak representing the ester and carboxyl groups.

0.033 mole of each of the starting materials and 0.41 gm. of sodium were dissolved in 250 cc. of methanol and electrolyzed between Pt electrodes with a current density of about 0.3 amp. per cm.^{2}. The reaction vessel was externally cooled with ice, and methanol was added to replace evaporative losses; periodic cleansing of the electrodes was also necessary. When the pH had risen above 7 an additional 0.033 mole of each of the starting materials was added and electrolysis was continued until the pH again rose above 7. A total of almost 0.2 faraday of current was passed.

The methanol solution was then poured off, leaving an insoluble viscous oil (mostly diphenyl eicosane), and evaporated completely. The product (a yellow oil) was extracted with ether three times and the combined ether extracts were extracted twice with 5 per cent Na_2CO_3 to remove unreacted starting material. The ether solution was washed with distilled water and evaporated completely, and the product was hydrolyzed by refluxing for 7 hours with ethanol (200 cc.), H_2O (15 cc.), and KOH (20 gm.). An additional 125 cc. of H_2O were added and the mixture was extracted twice with *n*-pentane (150 cc. aliquots) to remove any neutral compounds. The ethanolic-KOH solution was then acidified with HCl and extracted twice with 150 cc. of *n*-pentane. This procedure separated the desired reaction product (which was extracted into

pentane) from the 3,3,6,6-tetramethyl suberic acid, which remained in the ethanol-H_2O phase. The pentane was then evaporated and the product was distilled under high vacuum (0.001 mm. Hg), the fraction that distilled between 145° and 155° being collected. This resulted in approximately 2 gm. of a clear, colorless oil which remained liquid at 0° (mixture of two isomers). Analyses of the product indicated it to have the following composition:

Found: C 79.29, H 10.92, equivalent weight, 333

Calculated: C 79.46, H 10.91, equivalent weight, 332.5

50 mg. of DPMA was labeled with tritium by the Wilzbach method (8); this was performed by the New England Nuclear Corporation of Boston. Labile H^3 was removed by dissolving the tritiated acid in isooctane, extracting it into 0.05 N NaOH-alcohol (1:1), acidifying the OH^--alcohol, and reextracting into isooctane; this was repeated once. The tritiated acid was then purified by twice subjecting it to countercurrent distribution, using one of the systems of Ahrens and Craig (9) (upper phase, *n*-heptane; lower phase, acetonitrile-methanol-acetic acid). The central portion of the peak of the first countercurrent distribution (100 tubes) was rerun in 55 tubes, yielding a single peak which was symmetrical except for slight skewing on the right. The partition coefficient, calculated from the position of the peak, was 1.65 (23°).

B. Preparation of Solutions—An aqueous solution of sodium DPMA was prepared by weighing an aliquot of the acid, dissolving it in ethanol, adding an amount of NaOH just sufficient to neutralize the acid, evaporating the ethanol, and dissolving the fatty acid salt in freshly boiled distilled water. The concentration was verified by titration, with Nile blue in absolute ethanol as an indicator, according to the method of Gordon (1). Other solutions were made from the original concentrated one by dilution. A solution of the sodium salt of the tritiated acid was similarly prepared and its concentration was estimated from the concentration of radioactivity.

Palmitic acid-1-C^{14} was supplied by the Nuclear-Chicago Corporation; its specific activity was 20.5 mc. per mmole. An aqueous solution of sodium-1-C^{14}-palmitate was prepared as described above, and its concentration was determined from the concentration of radioactivity.

Solutions of the albumin-fatty acid anion complex were prepared for intravenous injection or infusion by adding to the fatty acid salt solution a small quantity of pooled rat serum, together with enough NaCl to make the solution isotonic. In the experiments involving the injection of tracer amounts of material in a small volume (1 cc. or less), 0.2 cc. of serum was added per cc. of fatty acid salt. In the experiments in which large volumes were infused, 0.1 cc. of serum was added per cc. of fatty acid salt.

C. Metabolic Experiments—Male Sprague-Dawley rats weighing approximately 250 gm. were used in these experiments. All rats were fasted 24 hours before use and throughout the time of the experiment. Except where otherwise indicated, intravenous injections of small volumes of tracer amounts of material were made rapidly into the tail vein. In the experiments in which relatively large amounts of fatty acid were infused intravenously, the solutions were administered slowly to avoid hemolysis. A small polyvinyl catheter (Suprenant Manufacturing Company, tubing type S-1) was inserted into the right femoral vein of a rat under ether anesthesia, and, when the animal had regained consciousness, the solution was infused at a constant rate of 4 to 6 cc. per hour by means of a constant infusion pump.

D. Radioassay—The C^{14} or tritium radioactivity of lipide fractions was measured by taking an appropriate aliquot in organic solvent to dryness under N_2, redissolving in 15 cc. of toluene containing 600 mg. of diphenyloxazole per 100 cc., and counting in a Packard Tri-Carb liquid scintillation spectrometer. Correction for quenching was made on the basis of a second count after the addition of a known amount of 1-C^{14}-palmitic acid or tritium-labeled Δ^4-cholestenone. $C^{14}O_2$ in Hyamine (Rohm and Haas, Philadelphia, Pennsylvania) was added to toluene-diphenyloxazole and counted in the same way. $C^{14}O_2$ produced in slice studies *in vitro* was collected in a KOH center well, converted to $BaCO_3$, and assayed with a windowless gas flow counter in the proportional region. Results were corrected to infinite thinness by using self-absorption data collected for the planchets and instrument used. The tritium content of water in the experiments *in vitro* was determined by distilling an aliquot of the medium under reduced pressure, adding 0.1 ml. of the distillate to a methanol-toluene-diphenyloxazole mixture, and counting in the liquid scintillation spectrometer.

RESULTS AND DISCUSSION

Binding of DPMA to Serum Albumin—The tightness of the binding of DPMA to human serum albumin was estimated by studying the competition between DPMA and palmitate for binding sites on the albumin molecule. A series of solutions was prepared as described in the study of fatty acid ion binding to serum albumin (2), each solution containing a constant amount of UFA-free human serum albumin (10), a small amount of 1-C^{14}-palmitate, and varying quantities of nonradioactive Na-DPMA, in a phosphate buffer with a pH of 7.45 and ionic strength of 0.16. 0.1 volume *n*-heptane was then added and, after equilibration, the C^{14} activity in the heptane phase was measured. By comparing the C^{14} activity in heptane in the presence of DPMA with the activity obtained without the addition of DPMA, it was possible to estimate how much extra palmitate would have had to be added to give an effect equivalent to that caused by the addition of DPMA to the solution. This estimation was achieved by using the results of the study of palmitate binding to serum albumin (2). The results are listed in Table I, in which the mole ratio of added palmitate to serum

TABLE I

Competition of DPMA and palmitate for binding to human serum albumin

Palmitate added	DPMA added	Amount of bound palmitate needed to obtain observed distribution of 1-C^{14}-palmitate
	moles/mole of albumin	
0.7	0	0.7
2.5	0	2.7
0.7	0.58	1.6
0.7	1.16	2.2
0.7	1.73	2.7
0.7	<2.3	3.0
0.7	<2.8	3.4

TABLE II

Disappearance of tritiated DPMA from plasma after intravenous injection in rats

Dosage	Time	Concentration of radioactivity in plasma lipides	Amount of dose remaining in plasma*
		c.p.m./ml.	%
Experiment A	10 min.	8,900	64
19.6 μM; 138,000 c.p.m.; intravenous infusion over 50 min. before time zero	62 min.	3,300	24
	3.0 hrs.	2,560	18
	5.9 hrs.	1,520	11
	22.9 hrs.	400	2.9
Experiment B	7 min.	10,500	59
0.7 μM; 177,000 c.p.m.; intravenous injection at time zero	73 min.	6,700	37
	3.2 hrs.	4,400	25
	6.1 hrs.	2,800	16
	23.1 hrs.	650	3.6

* Based on assumed total plasma volume of 10 ml.

TABLE III

Amounts of tritiated DPMA recovered after 23 hours and comparison with amounts of 1-C¹⁴-palmitate recovered

Body distribution	Percentage of dose recovered			
	Experiment A DPMA, 19.6 μM*	Experiment B DPMA, 0.7 μM*	Experiment E DPMA, 0.7 μM*	Experiment F Palmitate, 0.05 μM*
Total body (including intestinal contents)	60	70	63	25
Urine	−†	−	0.8	0.1
Feces	−	−	34	0.1
Tissue				
Liver	20	28	18	3
Adipose tissue	12	5	−	2
Skeletal muscle	7	4	−	10
Large intestine	−	−	7	−
Small intestine	−	−	5	−
Spleen	−	−	1	−
Kidney	<1	<1	−	<1
Heart	<1	<1	−	<1
Lung	−	−	−	1
Remainder of carcass	18	29	29	10
Serum	3	4	4	<1

* Fatty acids administered by injection or infusion.

† The symbol (−) means not examined

albumin is listed in the first column, the mole ratio of added DPMA to serum albumin in the second column, and the expected mole ratio of palmitate to albumin, if palmitate alone had been added, in the third column. Since the values listed in the third column are equal to or greater than the sum of the values in the first and second columns, it is apparent that DPMA binding to human serum albumin is at least as tight as palmitate binding, over the range studied. It has been assumed, in the subsequent studies, that similar results would have been obtained with rat serum albumin.

Rates of Disappearance from the Plasma—These are shown in Table II. 3 hours after administration of a tracer dose of labeled analogue (Experiment B), 25 per cent of the administered material remained in the plasma. Even after 23 hours there was still 3.6 per cent of the original dose in the circulating plasma. These results are to be contrasted with the considerably more rapid removal of normal fatty acids from the circulation. For example, Bragdon and Gordon (11) found that 10 minutes after a similar dose of 1-C¹⁴-palmitate to rats, only 1 per cent remained in the plasma. A similarly rapid removal of intravenously injected unesterified fatty acids has been observed in dogs and in humans by Fredrickson and Gordon (12). In these cases only about 1 per cent remained after 15 to 20 minutes.

A second rat (Experiment A, Table II) received 19.6 μM DPMA infused over a 50 minute period and the rate of disappearance was followed from the time the infusion ended. Because of the different experimental conditions the values for the early samples cannot be directly compared with those of Experiment B. However, at 6 and 23 hours the fraction retained in plasma is comparable in the two experiments, indicating that the fractional rate of removal was at least not markedly affected by the dosage in this range.

Body Distribution of the Tritiated DPMA—Body distribution administered in these two experiments and in an additional tracer experiment (Experiment E) was determined and compared with the distribution of 1-C¹⁴-palmitate (Experiment F). The data, shown in Table III, represent total radioactivity extracted by 1:1 alcohol-acetone. DPMA itself and any lipides derived from it would be included. As shown below, DPMA does not appear to be degraded by rat tissues; therefore there would be little or no tritium reincorporation into other compounds. It is probable, then, that virtually all of the radioactivity in these lipide extracts represents DPMA itself or DPMA incorporated into lipide complexes.

The most striking finding was the practically complete recovery of radioactivity in lipide form 23 hours after injection. 60 to 70 per cent was recovered in the carcass and, in Experiment E, an additional 34 per cent in the feces. In the latter experiment, 98 per cent of the administered dose was recovered in lipide form. In contrast, 23 hours after injection of a tracer dose of 1-C¹⁴-palmitate, only 25 per cent of the radioactivity remained in the carcass and only an additional 0.2 per cent could be recovered in urine and feces. The studies of McCalla *et al.* (13) have shown that 50 per cent of a tracer dose of 1-C¹⁴-palmitate given to fasted rats as the albumin complex is converted to $C^{14}O_2$ within 90 minutes. The present studies show that by 23 hours, 75 per cent of the administered dose has been either oxidized or converted to nonlipide metabolites. Some 75 per cent of the normal fatty acid can be metabolized in 23 hours; only 2 per cent at most of the DPMA was metabolized in the same time interval.

DPMA was found in all the tissues examined, as shown in Table III. The data shown for adipose tissue and for skeletal muscle are based on concentrations determined in epididymal fat and thigh muscle extrapolated to the total body fat and total body muscle, according to the values given by Castor *et al.* (14). Since the uptake in different fat depots and different muscle masses may vary, these results should be regarded only as approximations.

The largest fraction of DPMA was found in the liver, which contained approximately one-third of the amount remaining in the carcass. This again is very different from the results with 1-C¹⁴-palmitate. In the latter case only about one-eighth of the material remaining after 23 hours was found in the liver,

and the largest fraction was found in skeletal muscle. It is of interest to note that 10 minutes after injection of 1-C^{14}-palmitate Bragdon and Gordon found approximately equal quantities of radioactivity in liver and in skeletal muscle (15 to 20 per cent of the administered dose). Here, after 23 hours, the activity in the liver has decreased to a much greater extent than that in muscle, reflecting a more rapid turnover. Significant amounts of DPMA were found also in adipose tissue, skeletal muscle, and the intestines. Spleen, kidney, and heart contained very little.

The virtually complete recovery of DPMA radioactivity in lipide form implied that the compound was not degraded to any extent. This conclusion is supported by the finding that the main excretory route for DPMA is via the feces, with very little appearing in the urine (Table III). Bergström *et al.* (3) and Tryding and Westöö (4) have shown that 2,2-dimethyl-substituted long chain fatty acids are excreted predominantly in the urine as short chain (C-5 to C-7) dimethyl dicarboxylic acids. In contrast, 2,2,17,17-tetramethyl stearic acid, which apparently cannot be degraded by either β- or ω-oxidation, was excreted almost exclusively in the feces (5). The major fecal product was identified as the unchanged acid, and a small amount of 2,2,17,17-tetramethyl octadecanedioic acid was also demonstrated to be present. The excretion of DPMA primarily in the feces hence conforms to the results expected for a fatty acid which cannot be degraded by either β- or ω-oxidation.

Additional evidence for this conclusion was obtained from the studies *in vitro* summarized in Table IV. Release of tritium from DPMA to the water pool was taken as an index of its metabolism. The recovery of radioactivity in water after 4 hour incubations with liver slices was no greater than in the zero time control flask or in the incubated control containing no liver slices. Under the same conditions, as shown in Table IV, about 10 per cent of added 1-C^{14}-palmitate was metabolized to $C^{14}O_2$ in an equal time by liver slices. Labeled DPMA was also incubated with liver homogenates and no release of tritium to H_2O was demonstrable in 4 hours. In contrast, 23 per cent of 1-C^{14}-palmitate incubated for 4 hours with the same homogenate was converted to $C^{14}O_2$. These results, combined with the results *in vivo* described above, permit the conclusion that oxidative degradation of DPMA proceeds very slowly, if at all, in the rat. If it is assumed that the tritium in the compound is more or less randomly distributed throughout the structure, the completely negative results *in vitro* imply that all oxidative pathways are closed to this compound, including partial desaturation.

Chemical Form of Carcass Lipide Radioactivity—The lipide extracts of the whole carcasses of the rats in Experiments A, B, and F were analyzed to determine the chemical form of the radioactivity found therein. The method of Borgström (15) was employed to separate the lipides into three classes: neutral lipides, phospholipides, and unesterified fatty acids. Silicic acid columns were prepared as described by Hirsch and Ahrens (16), and the phospholipides were adsorbed on the column; all other lipides were eluted from the column with ethyl ether. The phospholipides were then eluted with methanol. The ether eluate was separated into neutral lipide and UFA fractions by means of an ion exchange column of Amberlite IRA 400. The three fractions were then separately analyzed for radioactivity.

The results of these analyses are presented in Table V. Comparison of the results in the two rats given DPMA shows them to be very similar, with 45 to 50 per cent of the activity present as UFA, and the remainder divided between neutral lipides and phospholipides. In contrast, the results with the rat given 1-C^{14}-palmitate show that only 8 per cent of the activity was in the UFA fraction, with 68 per cent present as phospholipide. The very high percentage of activity present as UFA in the DPMA rats after 24 hours suggests that this fatty acid is less available to esterifying enzymes than a normal fatty acid such as palmitate. This may be either a function of the specificity of the enzymes involved, or of the processes whereby fatty acids are made available at the sites of synthesis.

Intestinal Absorption of DPMA via the Lymph—1 cc. of olive oil containing approximately 1 μc. of tritiated DPMA was administered by stomach tube to a rat provided with a lymph fistula. The cannulation of the thoracic duct and the pre- and postoperative treatment of the rat was kindly carried out by Dr. J. Bragdon, who used the procedure described by Bergström *et al.* (17). The rat was permitted to ingest further olive oil *ad libitum* during the collection period. The lymph pro-

TABLE IV

Comparison of metabolism of DPMA and palmitate by rat liver slices

Slices, prepared with a Stadie-Riggs microtome, were incubated at 37° in 2 ml. of Krebs-Ringer phosphate buffer, pH 7.4. The medium contained bovine serum albumin (60 mg. per ml.) to which the labeled fatty acid substrate was bound.

Time	Wet weight of liver slices	Total radioactivity*	Substrate metabolized
Substrate: 1 μM DPMA containing 270,000 c.p.m.			
hrs.	*mg.*	*c.p.m.*	%
0	252	1,020	0.04
4	0	710	0.03
4	228	940	0.03
4	282	750	0.03
Substrate: 0.05 μM 1-C^{14}-palmitate containing 275,000 c.p.m			
4	247	23,400	8.5
4	237	27,800	10.5
4	241	25,900	9.4

* For DPMA, radioactivity recovered in water; 1-C^{14}-palmitate, radioactivity recovered in CO_2.

TABLE V

Chemical form of lipide-soluble radioactivity in rat carcasses 23 hours after administration of tritiated DPMA or 1-C^{14}-palmitate

Experiment	Fatty acid injections	Total radioactivity recovered*	Recovered radioactivity found as:		
			UFA	Neutral lipide	Phospholipide
		%	%	%	%
A	DPMA, 19.6 μM	99	48	33	19
B	DPMA, 0.7 μM	78	46	26	27
F	Palmitic acid, 0.05 μM	97	8	24	68

* Total recovery in analysis as percentage of total radioactivity fractionated.

duced during 24 hours was collected and an aliquot was extracted with alcohol-acetone (1:1). The feces excreted during the 24 hours was also collected and extracted with alcohol-acetone. 11 per cent of the administered radioactivity was recovered in the lipide extract of the lymph and 58 per cent in the feces. 31 per cent of the administered radioactivity was not accounted for by these analyses. It is highly probable that some of this was present in the intestinal contents of the rat at the end of the 24 hour period. It is also probable that some DPMA was absorbed via the portal blood vessels. In addition, the possibilities of lymph vessels bypassing the fistula, and of other potential errors suggested by Bergström *et al.* (17) must be kept in mind.

The lipide extract of the lymph was separated into neutral fat, phospholipide, and UFA fractions as described above, and the radioactivity in each fraction was determined. The total recovery of H^3 in this analysis was 93 per cent. Of the recovered radioactivity, 69 per cent was found in the neutral fat fraction, 25 per cent in the UFA fraction, and 6 per cent in the phospholipide fraction. This percentage of radioactivity found as UFA is distinctly higher than that obtained with normal long chain fatty acids (4 to 8 per cent (18)), or with either 2,2-dimethyl stearic acid (10 to 15 per cent (19)), or 2,2,17,17-tetramethyl stearic acid (10 to 15 per cent (5)). It thus appears that esterification during absorption is less complete with DPMA than with any of the other fatty acids listed.

Effect of DPMA Infusion on UFA Metabolism—The normal turnover of plasma unesterified fatty acids in the fasting animal is extremely rapid. After the intravenous injection of 1-C^{14} palmitate, plasma activity declines with an initial half-life of about 2½ minutes in the human subject (11, 20), and with an even shorter half-life in the dog (21) and the rat (11). Labeled CO_2 appears within minutes, reaching its peak specific activity in about 30 minutes in the human subject (12) and in a shorter time in the rat (13).

Experiments were conducted to ascertain the effect, on palmitate metabolism as UFA, of intravenously infusing a relatively large amount of DPMA. Two experimental and two control studies were performed. In each experimental study a fairly large amount of DPMA was infused over the course of about an hour. Directly following the infusion 1 cc. of 1-C^{14}-palmitate solution was rapidly injected through the catheter in the femoral vein and the rat was placed in a closed vessel fitted with air inflow and outflow tubes. Air was drawn through the vessel at the rate of 600 to 800 cc. per minute, and the CO_2 in the outflow air was collected in Hyamine as described by Fredrickson and Ono (22). The total CO_2 output was collected for 2 hours in a series of 5 to 15 minute collection samples, and then samples were collected intermittently at intervals of approximately an hour.

The control studies were performed in an identical fashion, except that the intravenous infusion given before the injection of 1-C^{14}-palmitate consisted of isotonic saline containing the same concentration of rat serum. The volume of the control infusion was identical with that of the respective experimental infusion.

The first experimental and control studies were performed with a modified desiccator with a volume of about 5 l. as the metabolic chamber. The second pair of studies was conducted with a small glass cylinder having a dead space of about 500 cc. The two pairs of studies are therefore not directly comparable and each experimental study should be compared only with its respective control. In the first experimental study 34 μeq. of DPMA were administered to the rat; in the second study 45 μeq. were administered.

The results of these studies are presented in Fig. 1, in which the cumulative C^{14} output as $C^{14}O_2$, plotted as the per cent of the total C^{14} dose administered, is plotted against time. As was expected from reports available in the literature (13), the appearance of $C^{14}O_2$ was extremely rapid in the control studies, being more rapid in the second control study because of the much smaller dead space in the metabolic chamber. The cumulative C^{14} output as $C^{14}O_2$ shown in Fig. 1 is, however, less than the percentage output in similar time periods previously reported for fasting rats (13). It is possible that the temporary expansion of extracellular fluid volume due to the relatively large intravenous infusion accounts in part for the smaller $C^{14}O_2$ outputs observed.

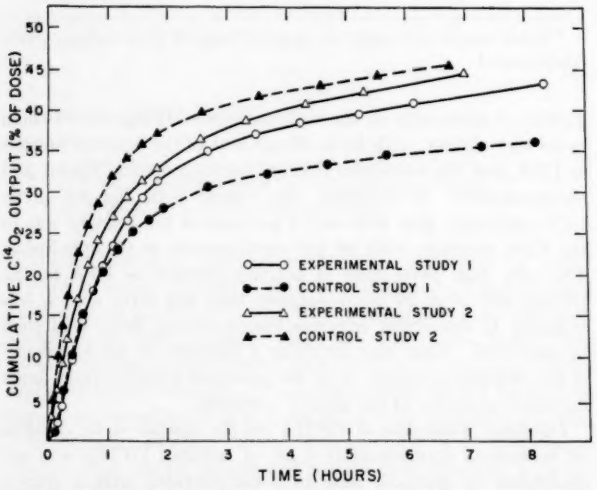

FIG. 1. Cumulative $C^{14}O_2$ output after intravenous 1-C^{14}-palmitate, as percentage of administered dose and effect of prior intravenous administration of DPMA. The rat in Experimental Study 1 received 34 μeq. of DPMA; the rat in Experimental Study 2 received 45 μeq.

Comparison of the results of the experimental studies with those of the controls indicates that DPMA had very little, if any, effect upon the rate of oxidation of the injected 1-C^{14}-palmitate. There is a suggestion of a very slight delay in the initial appearance of $C^{14}O_2$ in the experimental studies, as compared to the controls, but the differences are so slight that they might be due to biological variation alone. The normal content of UFA in the total plasma volume of a rat of this weight is about 6 μeq. The amount of DPMA infused into the experimental rats was, respectively, 5.7 and 7.5 times this amount. From the data on the disappearance of DPMA from the plasma (Table II) it may be inferred that the amount of DPMA in the plasma was more than twice the normal plasma UFA content for almost 30 minutes, and was at least as great as the normal plasma UFA content for more than 60 minutes. It therefore appears that the presence in plasma of a large quantity of a slowly turning over fatty acid which can compete with palmitate for binding to serum albumin does not significantly

interfere with the transport of the intravenously administered tracer dose of 1-C^{14}-palmitate to intracellular loci of oxidation.

These results, furthermore, show that the relatively large quantities of unesterified DPMA deposited in the tissues during the infusion and persisting largely in unesterified form (Table V) do not interfere with the transfer of labeled palmitate to the tissue sites of oxidation. The results of McCalla *et al.* (13) suggest that the UFA of plasma is in rapid isotopic equilibrium with an extravascular "pool" of fatty acid on the order of 100 times the size of the plasma pool. Even the larger dose of DPMA infused in the present study exceeded the plasma UFA pool only by a factor of 7.5. It may be that by the administration of considerably larger amounts of the analogue it will be possible to successfully block the tissue-binding sites which normally pick up UFA from serum albumin and thus effectively interfere with metabolism of serum UFA.

SUMMARY

1. The synthesis of a mixture of 3,3-dimethyl-13-phenylmyristic acid and 3,3-dimethyl-14-phenylmyristic acid (DPMA) has been described. Some of this material was tritiated by the Wilzbach method (8) and purified by countercurrent distribution.

2. The binding of DPMA to human serum albumin has been studied, relative to the binding of palmitate. In the range of 0 to 3 moles per mole of albumin, DPMA binding is at least as tight as that of palmitate.

3. When administered intravenously to rats, DPMA disappearance from the plasma was vastly slower than that of palmitate; even after 23 hours 3 to 4 per cent of the DPMA was still present in the plasma.

4. The body distribution of intravenously administered DPMA and the extent of its incorporation into several lipide fractions has been determined and compared with palmitate similarly administered. After 23 hours 60 to 70 per cent of the DPMA radioactivity was present in lipide form in the rat carcass, with about one-third of this found in the liver. In contrast, only 25 per cent of palmitate radioactivity was present in lipide form, with about one-eighth found in the liver. 45 to 50 per cent of the DPMA radioactivity was still present as unesterified fatty acid, whereas only 8 per cent of the palmitate was in this form. In one experiment an additional 34 per cent of DPMA radioactivity was recovered in the feces; in this experiment 98 per cent of the administered DPMA was recovered as lipide after 23 hours.

5. The almost complete recovery of DPMA after 23 hours, the mode of its excretion, and the results of experiments with liver slices and homogenates permit the conclusion that the process of oxidation of DPMA *in vivo* either proceeds negligibly slowly or does not occur at all.

6. 11 per cent of the DPMA radioactivity administered orally to a rat was recovered in the lymph in 24 hours. 25 per cent of this was present as unesterified fatty acid.

7. The effect on palmitate metabolism (as unesterified fatty acid) of intravenously infusing a relatively large amount of DPMA has been studied. It appears that the presence in plasma of a large quantity of a slowly turning over fatty acid which can compete with palmitate for binding to serum albumin does not significantly interfere with palmitate transport to intracellular loci of oxidation.

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The Enzymatic Synthesis of Sphingosine

II. FURTHER STUDIES ON THE MECHANISM OF THE REACTION

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(Received for publication, July 9, 1958)

The preparation of an enzyme system from brain tissue which catalyzes the biosynthesis of sphingosine has been described in a previous communication from this laboratory (1). The results of these investigations, as well as those obtained by other investigators from experiments performed *in vivo* (2, 3), indicated that carbon atoms 3 and 2 of serine are the source of carbon atoms 1 and 2 of sphingosine. Palmityl-CoA was a precursor of carbon atoms 3 to 18 of sphingosine. The biosynthesis of sphingosine required the presence of TPNH when serine and palmityl-CoA were used as substrates. The enzyme system used was found to catalyze the reduction of palmityl-CoA in the presence of TPNH. These observations suggested that the carbon-carbon condensation might occur between palmitic aldehyde and a 2-carbon fragment derived from serine. The possibility could not be excluded in the earlier experiments that the condensation occurred by way of a thiohemiacetal derivative of palmitic aldehyde with CoA. The present studies demonstrate that palmitic aldehyde in the absence of CoA can serve as a precursor of sphingosine. The requisite activation of the methylene carbon atom of serine is obtained by the formation of a Schiff base-metal complex of serine with pyridoxal-P and Mn^{++} ions. Ethanolamine does not readily form a complex with pyridoxal and metal ions and this finding may explain in part the observation that ethanolamine does not participate in the biosynthesis of sphingosine.

EXPERIMENTAL

The enzyme system was prepared from cell-free preparations of brain tissue obtained from 12- to 18-day-old rats. The particles which sedimented between 20,000 and 100,000 $\times$ *g* were washed two times with 0.1 M potassium phosphate buffer (pH 7.8) and resuspended in the same solution or in 0.05 M potassium phosphate buffer (pH 7.4). The particles which were resuspended in the 0.05 M phosphate buffer were subjected to sonic oscillation at 9 kc. for 10 minutes at $-2°$ and the resulting mixture was centrifuged at 100,000 $\times$ *g* for 1 hour.

Sphingosine was recovered from the incubation mixtures by the extraction procedures described previously (1). When DL-serine-3-C^{14} was incubated with the enzyme system obtained by sonic vibration of the particulate material, an additional purification of the recovered sphingosine was required in order to eliminate the radioactivity in the control experiments. Satisfactory decontamination was obtained by adsorption of the sphingosine on a silicic acid column and elution with a 2:1 chloroform-methanol solution (Table I).

Palmitic aldehyde was purchased from K and K Laboratories, Long Island City, New York. It was redistilled immediately before use under vacuum in the presence of a small crystal of zinc chloride. The freshly distilled material was dissolved in anhydrous heptane. The heptane was evaporated from an appropriate aliquot under nitrogen, and the palmitic aldehyde was suspended in a 10 per cent solution of bovine serum albumin. Alternately, the palmitic aldehyde was taken up in Tween 20 (polyoxyethylene sorbitan monolaurate, Atlas Powder Company) followed by the addition of the required amount of water.

Ethanolamine-1,2-C^{14} hydrochloride was purchased from the Isotopes Specialties Company, Inc. L-Serine-U-C^{14} was purchased from Schwarz Laboratories, Inc., and DL-serine-3-C^{14} was obtained from Nuclear-Chicago Corporation. Palmityl-CoA was prepared enzymatically (4) or synthesized from palmitoyl chloride by a method kindly furnished by Dr. Werner Seubert. Phenazine methosulfate was a gift from Dr. D. E. Green.

RESULTS

The possibility existed that the failure to demonstrate the incorporation of palmitic aldehyde into sphingosine in previous experiments (1) was due to improper solubilization of the aldehyde. An inhomogeneous distribution of palmitic aldehyde was observed when suspensions of this material in solutions of serum albumin were treated with Schiff's reagent. A uniform dispersion of palmitic aldehyde was obtained with the use of suitable concentrations of the surface-active agent, Tween 20. A study was therefore undertaken to determine the effect of varying the concentration of Tween 20 on the enzymatic synthesis of sphingosine. The particulate enzyme system was only slightly inhibited by the addition of 0.6 mg. of Tween 20 per ml. of incubation mixture, whereas higher levels drastically impaired the incorporation of serine-C^{14} into sphingosine (Table II).

The enzyme system obtained in the supernatant solution after sonic oscillation of the particulate preparation catalyzed the incorporation of palmitic aldehyde suspended in a solution of serum albumin into sphingosine (Table III, Experiment 1). The conversion of palmitic aldehyde to sphingosine was therefore reexamined in the particulate enzyme preparations with the use of the minimal amount of Tween 20 required to disperse adequately the long chain aldehyde. Experiments performed

TABLE I

Recovery of sphingosine from silicic acid column

21 μmoles of sphingosine in 0.5 ml. of 2:1 chloroform-methanol solution were applied to a 15 × 65-mm. silicic acid column and eluted as indicated.

Eluent	Volume	Recovered sphingosine	
	ml.	*μmoles*	*%*
Chloroform	30	0	0
Chloroform-methanol:			
50:1	30	0	0
10:1	30	3.5	17
2:1	30	17.0	81
Methanol	30	1.5	7

with 0.6 mg. of Tween 20 per ml. of incubation mixture revealed that the washed particulate preparations from brain tissue catalyzed the conversion of palmitic aldehyde to sphingosine under these conditions (Table III, Experiments 2 and 3).

The addition of DPN or CoA to the incubation medium caused a marked reduction in the conversion of palmitic aldehyde to sphingosine (Table IV). Supplemental TPN exerted a much smaller effect in this respect. A likely explanation for the inhibitory effect of DPN was found in experiments in which the oxidation of palmitic aldehyde in the presence of DPN was observed to be catalyzed by an enzyme present in the washed particles from rat brain tissue (Fig. 1). TPN was much less effective in this respect, particularly in the absence of supplemental CoA (Fig. 2). The inhibition caused by the addition of CoA may be due to the formation of a thiohemiacetal derivative of palmitic aldehyde with CoA. Such a compound may be subsequently oxidized to palmityl-CoA (1).

The washed particulate enzyme preparations exhibited marked serine decarboxylase activity which had a clear dependence upon the presence of pyridoxal-P (Table V). The addition of palmityl-CoA and TPNH exerted relatively little effect upon the decarboxylation of serine. In order to investi-

TABLE II

Effect of Tween 20 on conversion of serine-C^{14} to sphingosine

The reaction mixtures contained 150 μmoles of potassium phosphate buffer (pH 7.8), 2 μmoles of L-serine-U-C^{14} (2.0 μc.), 1 μmole of pyridoxal-P, 20 μmoles of glucose-6-P, 2 mg. of glucose-6-P dehydrogenase (2.0 units (Kornberg (5)) per mg. of protein), 5 μmoles of $MgCl_2$, 0.5 μmole of $MnCl_2$, 0.3 μmole of TPN, 0.6 μmole of DPN, 2 μmoles of cytidine diphosphate-choline, and 20 μmoles of nicotinamide. The enzyme (20 mg. of protein) was obtained by resuspending the twice-washed particles which sedimented between 20,000 and 100,000 × *g*. The total volume was 2.0 ml., and the incubation time was 4 hours at 37° in air. After extracting the sphingolipides with butanol, 15 μmoles of sphingosine were added to each sample as carrier.

Additions	Radioactivity of recovered sphingosine
	c.p.m./μmole
None	8
1.2 mg. of Tween 20	16
0.6 μmole of CoA + 4 μmoles of ATP	535
+ 1.2 mg. of Tween 20	430
+ 6.0 mg. of Tween 20	50

TABLE III

Effect of palmityl CoA and palmitic aldehyde on conversion of serine-C^{14} to sphingosine

The incubation mixtures contained 150 μmoles of potassium phosphate buffer (pH 7.8), 4 μmoles (4.0 μc.) of DL-serine-3-C^{14} in Experiment 1, 2 μmoles (2.0 μc.) of L-serine-U-C^{14} in Experiments 2 and 3, 1 μmole of pyridoxal-P, 5 μmoles of $MgCl_2$, 0.5 μmole of $MnCl_2$, and 2 μmoles of cytidine diphosphate-choline. The enzyme (2.0 mg. of protein) used in Experiment 1 was contained in the supernatant solution after sonic oscillation. The enzyme system for Experiments 2 and 3 (15 mg. of protein) was obtained by resuspending the washed particles from brain tissue and was supplemented by the addition of 20 μmoles of nicotinamide. The total volume was 2.0 ml., and the incubation time was 3 hours at 37° in air. Sphingosine was recovered as described in Table II.

Experiment No.	Additions	Radioactivity of recovered sphingosine
		c.p.m./μmole
1	None	0
	0.12 μmole of palmityl-CoA + 1 μmole of TPNH	117
	0.12 μmole of palmitic aldehyde + 10 mg. of serum albumin	87
2	None	37
	0.12 μmole of palmityl-CoA + 1 μmole of TPN*	293
	0.12 μmole of palmitic aldehyde + 1.2 mg. of Tween 20	296
3	None	16
	0.32 μmole of palmityl-CoA + 1 μmole of TPN*	558
	0.12 μmole of palmitic aldehyde + 1.2 mg. of Tween 20	180

* These reaction mixtures also contained 20 μmoles of glucose-6-P, 2 mg. of glucose-6-P, dehydrogenase, 0.6 μmole of DPN.

TABLE IV

Effect of pyridine nucleotides and CoA on conversion of palmitic aldehyde to sphingosine

The incubation mixtures contained 150 μmoles of potassium phosphate buffer (pH 7.8), 2 μmoles (2.0 μc.) of L-serine-U-C^{14}, 1 μmole of pyridoxal-P, 5 μmoles of $MgCl_2$, 0.5 μmole of $MnCl_2$, 2 μmoles of cytidine diphosphate-choline, 1.2 mg. of Tween 20, and 20 μmoles of nicotinamide. The enzymes (15 mg. of protein) were obtained in the washed particulate preparation. The incubation conditions and recovery of sphingosine were the same as described in Table III.

Additions	Radioactivity of recovered sphingosine in experiment	
	1	2
	c.p.m./μmole	
None	37	16
0.12 μmole of palmitic aldehyde	296	180
+ 0.6 μmole of DPN	14	10
+ 0.6 μmole of CoA	39	54
+ 0.3 μmole of TPN	160	130

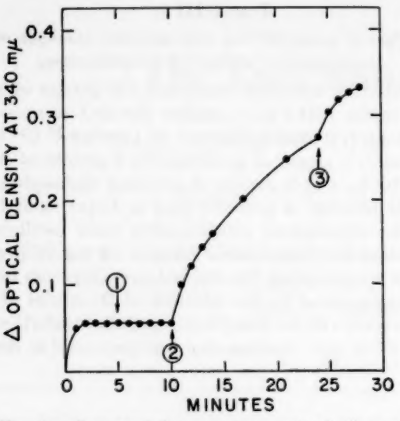

FIG. 1. Spectrophotometric measurement of the reduction of DPN by palmitic aldehyde. The reaction mixtures contained 120 μmoles of potassium phosphate buffer (pH 7.8), 5 μmoles of $MgCl_2$, and washed, resuspended particles sedimenting between 20,000 and 100,000 × *g* (2.5 mg. of protein) in a total volume of 1.5 ml. The reaction was initiated by the addition of 1 μmole of DPN. Additions at *Arrow 1*, 1.0 mg. of Tween 20; *Arrows 2* and *3*, 0.1 ml. of an aqueous solution of palmitic aldehyde (1×10^{-3} M) containing 10 mg. of Tween 20 per ml. The temperature was 22°.

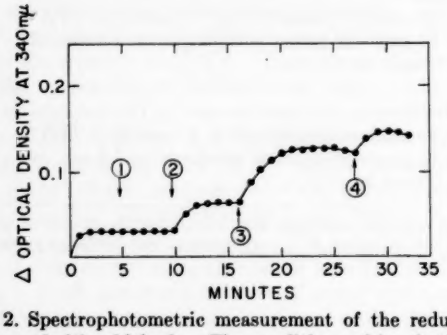

FIG. 2. Spectrophotometric measurement of the reduction of TPN by palmitic aldehyde. The conditions of incubation were the same as those for Fig. 1. The reaction was initiated by the addition of 1 μmole of TPN. Additions at *Arrow 1*, 1.0 mg. of Tween 20; *Arrows 2* and *4*, 0.1 ml. of the solution of palmitic aldehyde (1×10^{-3} M) and Tween 20 (10 mg. per ml.); at *Arrow 3*, 0.2 μmole of CoA was added.

gate the possibility that ethanolamine might be a precursor of sphingosine, experiments were conducted which compared the conversion of labeled ethanolamine and serine to sphingosine (Table VI). The failure of this enzyme system to incorporate labeled ethanolamine into sphingosine is in accord with previous studies performed with labeled ethanolamine *in vivo* (2, 3). This observation may be explained in part by the relative inability of ethanolamine to form an appropriate Schiff base-pyridoxal-P-metal complex as readily as serine. A direct spectrophotometric comparison (7) of the formation of the respective derivatives of serine and ethanolamine with pyridoxal and Ni^{++} is shown in Fig. 3. The data indicate that a Schiff base-metal complex of serine with pyridoxal and Ni^{++} is quite rapidly produced, whereas ethanolamine does not readily form such a complex at the concentrations used in these experiments.

TABLE V

Decarboxylation of L-serine-U-C^{14}

Subcellular particles of rat brain tissue (12 mg. of protein) which sedimented between 20,000 and 100,000 × *g* were washed two times and resuspended in 0.1 M potassium phosphate buffer (pH 7.8) and placed in the main compartment of a double-armed Warburg vessel, along with 2 μmoles (2.0 μc.) of L-serine-U-C^{14} 5 μmoles of $MgCl_2$, 0.5 μmole of $MnCl_2$, and 20 μmoles of nicotinamide. After incubation for 1 hour at 37°, 1 μmole of $KHCO_3$ was tipped into the main compartment from side arm 1, and following this addition, 0.5 ml. of 5 N H_2SO_4 was introduced from side arm 2. The liberated $C^{14}O_2$ was allowed to diffuse for 30 minutes with shaking and was recovered in 0.3 ml. of 0.8 M Hyamine* in methanol (6) contained in the center well. The radioactivity was determined with the use of a liquid scintillation spectrometer.

Additions	Pyridoxal-P	Radioactivity of recovered CO_2
	5×10^{-4} M	*c.p.m.*
None	+	178,000
None	−	5,500
0.1 μmole of palmityl-CoA + 1 μmole of TPNH	+	190,000
0.1 μmole of palmityl-CoA + 1 μmole of TPNH	−	6,800

* *p*-Diisobutyl cresoxy ethoxy ethyl dimethyl benzyl ammonium chloride, Rohn and Haas Co.

TABLE VI

Comparison of utilization of L-serine-C^{14} and ethanolamine-C^{14} for enzymatic synthesis of sphingosine

The incubation mixtures contained 150 μmoles of potassium phosphate buffer (pH 7.8), 2 μmoles (2.0 μc.) of labeled serine or ethanolamine as indicated, 1 μmole of pyridoxal-P, 5 μmoles of $MgCl_2$, 0.5 μmole of $MnCl_2$, and washed particles (15 mg. of protein). The incubation conditions and recovery of sphingosine were the same as described in Table III.

Substrate	Additions	Radioactivity of recovered sphingosine experiment	
		1	2
		c.p.m./μmole	
L-Serine-U-C^{14}	None	25	23
	0.16 μmole of palmityl-CoA + 1.3 μmoles of TPNH	327	468
Ethanolamine-1,2-C^{14}	None	6	31
	0.16 μmole of palmityl-CoA + 1.3 μmoles of TPNH	7	32

Furthermore, a reaction between serine and pyridoxal-P may be readily detected photofluorometrically,[1] whereas ethanolamine was inactive in this respect as well. Although relatively large amounts of $C^{14}O_2$ were produced from L-serine-U-C^{14}, only very small amounts of ethanolamine could be detected in such experiments.[2] Thus, extensive decomposition of the Schiff base-metal complex is probably occurring in these systems (8).

[1] R. O. Brady and R. W. Albers, unpublished data.

[2] Dr. Alan Burkhalter, personal communication.

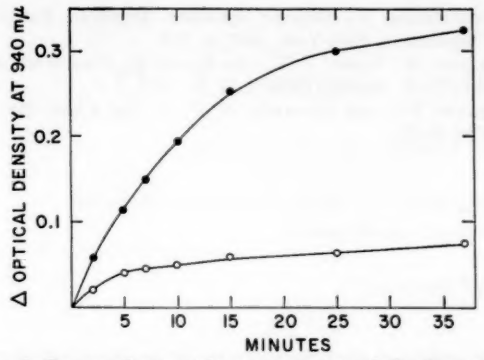

FIG. 3. Spectrophotometric measurement of the formation of the nickel complexes with the Schiff base derivatives of serine (●) or ethanolamine (○) and pyridoxal. The reaction mixtures contained 27 μmoles of pyridoxal, 27 μmoles of L-serine or ethanolamine, and 13.5 μmoles of nickel nitrate in a total volume of 0.9 ml., pH 7.5. The temperature was 23°.

Evidence obtained in earlier experiments (1) indicated that dihydrosphingosine was probably a precursor of sphingosine. Data were obtained which suggested that the enzyme preparation used in these experiments could catalyze the desaturation of dihydrosphingosine. Experimental confirmation for such a reaction was obtained in the following manner: 1.5 μmoles of dihydrosphingosine were suspended in 15 mg. of Tween 20 and incubated with the particulate enzyme system obtained from rat brain tissue (75 mg. of protein) in 15 ml. of 0.1 M potassium phosphate buffer (pH 7.8) containing 30 μmoles of $MgCl_2$, 3 μmoles of $MnCl_2$, 100 μmoles of nicotinamide, 1 μmole of DPN, 1 μmole of TPN, and 5 mg. of phenazine methosulfate at 37° for 6 hours under oxygen. The recovered sphingosine was separated from dihydrosphingosine with the use of paper chromatography (1). It was subsequently eluted from the paper and quantitatively analyzed (9). Under the conditions described, 87 mμmoles of dihydrosphingosine were converted to sphingosine.

DISCUSSION

Previous studies indicated that rat brain tissue contains an enzyme which catalyzes the reduction of palmityl-CoA in the presence of TPNH (Reaction 1). The present experiments

$$CH_3(CH_2)_{14}\overset{\overset{O}{\|}}{C}\text{—SCoA} + TPNH + H^+ \rightleftharpoons CH_3(CH_2)_{14}CHO + CoASH + TPN^+ \quad (1)$$

demonstrate that a Schiff base-metal complex is rapidly formed with serine, pyridoxal, and Ni^{++} ions, and that ethanolamine does not readily yield such a complex. These data, as well as the photofluorometric detection of a reaction between serine and pyridoxal-P, suggest that serine forms a complex with pyridoxal-P and Mn^{++} ions (Reaction 2). The formation of such a complex results in the activation of the methylene group at carbon atom 2 of serine. It is likely that the presence of the carboxyl group of serine contributes to the activation of the

$$HOCH_2CH(NH_2)COOH + Mn^{++} + \text{pyridoxal-}PO_4 \rightleftharpoons \left[\begin{array}{l}\text{COOH}\\ \ominus\,|\,Mn^{++}\\ HOCH_2C\text{—N=pyridoxal-}PO_4\end{array}\right] + H^+ \quad (2)$$

methylene carbon of serine (10). The resonance-stabilized carbanion can then participate in a carbon to carbon addition reaction. It is probable that the condensation reaction occurs because of the positive dipole moment at carbon atom 1 of the palmitic aldehyde (Reaction 3). The reaction for the biosynthesis of sphingosine bears a strong resemblance to an aldol

$$\left[\begin{array}{l}\text{COOH}\\ \ominus\,|\,Mn^{++}\\ HOCH_2C\text{—N=pyridoxal-}PO_4\end{array}\right] + CH_3(CH_2)_{14}\overset{\overset{O^{4-}}{\|}}{\underset{\underset{H}{|}}{C}}{}^{4+} \rightarrow CH_3(CH_2)_{14}CH(OH)CH(NH_2)CH_2OH + \text{pyridoxal-}PO_4 + Mn^{++} + CO_2 \quad (3)$$

condensation of the Knoevenagel type and may represent a novel mechanism for the biological elongation of carbon chains (11).

The present experiments have demonstrated the enzymatic conversion of dihydrosphingosine to sphingosine (Reaction 4). The desaturation of dihydrosphingosine may be required for the biosynthesis of complex sphingolipides such as sphingomyelin (12).

$$CH_3(CH_2)_{12}CH_2CH_2CH(OH)CH(NH_2)CH_2OH + \text{flavin} \rightarrow CH_2(CH_2)_{12}CH{=}CHCH(OH)CH(NH_2)CH_2OH + \text{flavin}\cdot 2H \quad (4)$$

SUMMARY

The enzymatic synthesis of sphingosine seems to occur by a reaction involving the addition of palmitic aldehyde to the activated methylene carbon atom 2 of serine in the presence of pyridoxal phosphate and Mn^{++}.

Ethanolamine is not a precursor of sphingosine and does not readily form a Schiff base-metal complex with pyridoxal and Ni^{++} ions.

The enzyme preparations used in these studies catalyzed the oxidation of palmitic aldehyde in the presence of diphosphopyridine nucleotide.

The conversion of dihydrosphingosine to sphingosine has been observed in an enzyme system obtained from rat brain tissue.

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The Enzymatic Synthesis of Inositol Phosphatide*

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(Received for publication, June 12, 1958)

Since the isolation of myo-inositol from biological sources over a hundred years ago, little has been reported concerning its metabolism. Experiments in nutrition have implicated inositol variously as a vitamin (1) and as a lipotropic agent (2); and more recently inositol has been identified as a soluble factor required for the survival of human cells grown in tissue culture (3). The presence of inositol lipides in mammalian tissue in the form of diphosphoinositide (4) and monophosphoinositide (inositol phosphatide) (5) has been established. Recently, several investigators have confirmed the observation that the exchange of inorganic P^{32} with the phospholipides of tissue slices and homogenates occurs at a greater rate in the inositol lipide fraction than in the ethanolamine-, choline-, or serine-containing fractions (6–9).

In the present study, the metabolism of inositol was investigated by means of a spectrophotometric method for the determination of inositol and by the use of tritium-labeled inositol. A particulate preparation obtained from lyophilized guinea pig kidney mitochondria which catalyzed the incorporation of inositol into inositol phosphatide in the presence of cytidine nucleotide and Mg^{++} was examined in detail.

EXPERIMENTAL

Myo-inositol and scyllo-inosose were purchased from the Nutritional Biochemicals Corporation. Nucleotides were obtained from the Pabst Laboratories and from the Sigma Chemical Company, and Tween 20 (polyoxyethylene sorbitan monolaurate) from the Atlas Powder Company. Hyamine (*p*-diisobutyl cresoxy ethoxy ethyl dimethyl benzyl ammonium chloride) was purchased from Rohm and Haas Company. Inositol phosphatide was the gift of Dr. Elwood Titus. D-α,β-Dimyristin, dimyristoyl-L-α-glycerophosphoric acid, D-α,β-dioleein and dioleoyl-L-α-glycerophosphoric acid were the generous gift of Dr. Erich Baer. Sphingomyelin was purchased from L. Light and Company, Ltd. Cytidine diphosphate glycerol (10) was the gift of Dr. J. Baddiley. Sodium *m*-periodate was purchased from the G. Frederick Smith Chemical Company. Barium hydroxide, 0.3 N, and 5 per cent zinc sulfate deproteinizing reagents were prepared as described by Nelson (11).

Preparation of Labeled Compounds—Inositol-2-H^3 was prepared from scyllo-inosose by the method described by Posternak (12) with the exception that H^3OH was substituted for D_2O. The final product contained a small amount of platinum and had a melting point of 224°; the mixed melting point was 224°. Microanalysis was as follows:

$C_6H_{12}O_6$

Calculated:	C 40.00, H 6.71
Found (corrected for residue):	C 40.09, H 6.82

Inositol-H^3 (randomly labeled) was prepared by the method of Wilzbach (13). 1 gm. of inositol was exposed to 2.0 curies of tritium gas for 2 weeks at room temperature. The product was lyophilized and recrystallized to constant specific activity. The purified inositol was completely stable and had a specific activity of 15 μc. per μmole. Tritium-labeled inositol and lipides were counted in steel planchets in a gas flow Geiger counter. The material to be counted was pipetted onto a planchet which was then flooded with ethanol and dried alternately three times in order to obtain uniform distribution of the sample. Satisfactory reproducibility and a counting efficiency of about 30 per cent was observed with samples weighing less than 10 μg. in an area of 8 cm^2. Self-absorption was measured by means of an internal standard (14). Reproducibility was poor when the self-absorption was over 60 per cent. Radioactivity in proteins was detected in a liquid scintillation counter. CMP was also labeled by the method of Wilzbach. A vial containing 40 mg. of CMP (sodium salt, Sigma Chemical Company) was exposed to 2 curies of tritium gas for 1 week. The product contained 245 mc. of tritium. Solution in water followed by evaporation reduced the total radioactivity to 14 mc. The product was then adsorbed on a column of Dowex 1-formate and was eluted with 0.1 M formic acid. The eluate was evaporated to dryness and subjected to distribution in the two phases of chloroform-methanol-water, 1:1:1, as described below.

Determination of Inositol—The reaction of inositol with sodium periodate was measured. The reduction of periodate was observed spectrophotometrically (15). A sample containing 0.01 to 0.3 μmole of free inositol in 1.0 ml. was mixed with 1.0 ml. of 0.3 N barium hydroxide and heated at 100° for 15 minutes. This treatment was sufficient to oxidize sugars present to acidic derivatives. 1 ml. of 5 per cent zinc sulfate was then added. The mixture was centrifuged and 2.0 ml. of the resulting supernatant solution were treated with either a slurry or a short column of Amberlite IRA-400 (OH) (about 1 ml. of packed wet resin). When a column was used, 2.0 ml. of water were also passed through it and the combined eluate was evaporated to a volume of 1.0 ml. When the slurry was employed, 1.0 ml. of the supernatant fluid was used for the estimation. The slight amount of dilution introduced by the water in the resin was

* A preliminary report of this investigation was presented before the American Society of Biological Chemists at its Forty-ninth Annual Meeting at Philadelphia, April, 1958.

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consistent and was compensated for by an inositol standard taken through the same procedure.

1.0 ml. of eluate was added to 1.0 ml. of 1 M potassium acetate buffer, pH 4.7, in a 4.0 ml. silica cuvette, followed by 0.3 ml. of 0.01 M sodium *m*-periodate. A spectrophotometric reading was immediately taken at 260 mμ. The reaction was allowed to continue at room temperature until there was no further decrease in optical density. This step generally took 20 to 30 minutes. The decrease in optical density represented oxidation of glycerol. Inositol was not oxidized under these conditions. The reaction mixture was then heated in the dark at 65° for 2 hours, cooled, and read again. The difference in the optical densities before and after heating was due to oxidation of inositol. 6 equivalents of periodate were consumed per equivalent of inositol (Fig. 1). A linear relationship between optical density and concentration was observed (Fig. 2) when less than two-thirds of the periodate was consumed. Very little formaldehyde was produced. Since oxidation of inositol yields essentially only formic acid, the glycerol content could be estimated at this point by means of chromotropic acid determination of the formaldehyde present (16). A somewhat different technique was used for the determination of total free and combined inositol. The sample was first hydrolyzed with 6 N HCl for 48 hours (4), evaporated to dryness, and then passed through a mixed column of Amberlite IR-120 and IRA-400 (OH). Glycerol was decomposed by heating at 180° for 2 hours. The sample was then dissolved in water and passed through the same column, evaporated to a volume of 1.0 ml., and treated with periodate as above. Good agreement with the microbiological assay was obtained[1].

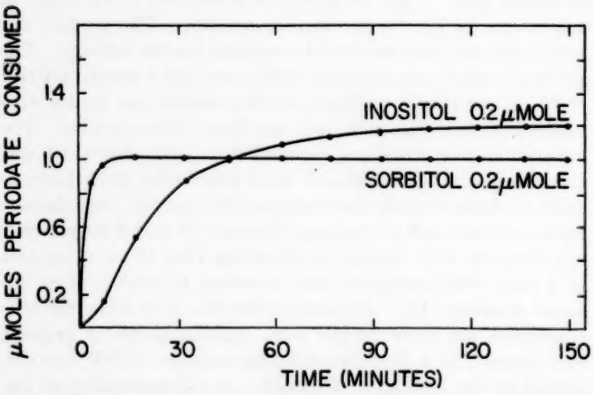

FIG. 1. Time course and stoichiometry of periodic oxidation of inositol and D-sorbitol at pH 4.7 and 65°. 5 μmoles of periodate were reduced per μmole of sorbitol, and 6 μmoles of periodate were reduced per μmole of inositol.

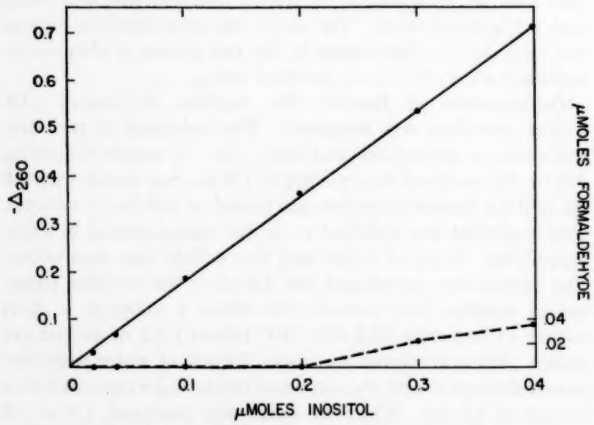

FIG. 2. Oxidation of inositol by sodium periodate. ——, change in optical density as a function of the amount of inositol in the oxidation mixture; ----, formaldehyde detected.

Preparation of Enzyme System—Four to five 200 gm. NIH-strain male guinea pigs were decapitated. The kidneys were homogenized in 4 volumes of a solution containing 0.13 M KCl, 0.003 M $MgSO_4$, and 0.012 M potassium phosphate buffer, pH 7.4. The cell-free preparation was centrifuged at 600 × *g* for 1 minute and the overlying suspension was centrifuged at 7500 × *g* for 20 minutes. The sedimented particles were resuspended in a volume of the KCl-$MgSO_4$-phosphate buffer equal to that of the discarded supernatant solution and recentrifuged. This washing procedure was repeated. The preceding steps were all performed at 2°. The sedimented fraction was resuspended in a small volume of water and lyophilized. The dried preparation was stored at −22° under vacuum. Enzymatic activity diminished slowly over a period of 2 weeks. The dried residue was prepared for incubation by trituration with 100 volumes of the KCl-$MgSO_4$-phosphate solution, washing twice in the buffer by centrifugation, and finally resuspension in the buffer to give a concentration of 15 mg. per ml. of the lyophilized preparation. All incubations were performed at 37° under nitrogen.

Isolation of Lipides—Phospholipides were isolated by a modification of the method of Dawson (17). 1 ml. of 1 per cent bovine serum albumin was added to 0.3 ml. of incubation mixture followed by 2.0 ml. of cold 20 per cent trichloroacetic acid. The mixture was filtered through Whatman No. 42 paper in a Buchner funnel and refiltered until the filtrate was clear. The precipitate was washed with 50 ml. of cold 10 per cent trichloroacetic acid and finally with 100 ml. of cold water. The filter paper containing the precipitate was transferred to a 10 × 50 mm. Soxhlet thimble and dried under vacuum for 1 hour. The thimble was then extracted with 15 ml. of chloroform-methanol, 1:1, for 3 hours in a micro-Soxhlet apparatus. From 0.1 to 0.2 ml. of the extraction mixture was plated and counted as described.

RESULTS

Uptake and Release of Inositol by Tissue Preparations—Many tissue slice preparations, particularly brain and testis, released inositol into the medium upon incubation. This release was depressed by the addition of exogenous inositol. Only in preparations of kidney tissue slices was it possible to demonstrate a net decrease in free inositol in the incubation medium (Table I). This tissue was therefore selected for further studies with isotopic inositol.

Disposition of Inositol-2-H³ in Vivo—After the injection of inositol-2-H³ into a rat, radioactivity was detected in trichloroacetic acid-soluble and trichloroacetic acid-insoluble tissue fractions. The acid-soluble fractions contained inositol and a radioactive anionic component which did not contain inositol. The acid-insoluble fractions contained a component extractable with methanol-chloroform as well as an unextractable component.

[1] H. Eagle and B. W. Agranoff, unpublished observations.

TABLE I

Change in inositol concentration of incubation medium

250 mg. of guinea pig tissue were incubated in the presence of added inositol as indicated, in 2.5 ml. of Krebs-Ringer-bicarbonate buffer containing 10 μmoles of glucose, at 37°, with shaking under 95 per cent O_2-5 per cent CO_2. At the end of 2 hours the mixture was chilled, centrifuged, and the supernatant solution analyzed for free inositol.

Preparation	Duration of incubation	Inositol added	Total inositol recovered from incubation medium	Net change in inositol
	hrs.	*μmoles*	*μmoles*	*μmoles*
Brain mince	0	1	2.01	+0.54
	2	1	2.55	
	0	0	0.98	+0.79
	2	0	1.77	
Heart slice	0	1	1.31	−0.02
	2	1	1.29	
	0	0	0.28	+0.18
	2	0	0.46	
Kidney slice	0	1	1.44	−0.63
	2	1	0.81	
	0	0	0.39	+0.02
	2	0	0.41	
Liver slice	0	1	1.24	+0.01
	2	1	1.25	
	0	0	0.30	+0.10
	2	0	0.40	
Testis mince	0	1	2.74	+0.89
	2	1	3.63	
	0	0	1.76	+0.91
	2	0	2.67	

The distribution of the latter three components in various organs is shown in Table II. In similar experiments, the acid-insoluble fractions were first extracted with ether, then ethanol, and finally with chloroform-methanol (1:1). Although this technique did not permit a quantitative separation of classes of lipides, striking differences were noted in solubility of lipides from different organs. About 70 per cent of the radioactivity in the kidney lipide was recovered in the ethanol-soluble fraction, whereas 90 per cent of the radioactivity recovered from liver was in the ether-extractable fraction. In each of the three categories listed in Table II, the greatest incorporation per gm. of tissue was in the kidney. Isolation of total inositol as described in "Experimental" revealed that the radioactivity present in the kidney lipide was present as inositol. The total radioactivity recovered from all of the fractions represented 2.28 per cent of the inositol given by injection.

Identification of Anionic Material—The anionic component of the trichloroacetic acid-soluble fraction was isolated as a single peak by gradient elution with formic acid from a Dowex 1-formate column. The supernatant solution obtained after centrifugation of rat or guinea pig kidney at 100,000 × *g* for 30 minutes was found to contain enzymes which readily catalyzed the conversion of inositol to the anionic product. The system was inhibited slightly by UTP, CTP, and ATP, and was unaffected by iodoacetate or KCN, or by preincubation with glucose and

TABLE II

Incorporation of inositol-2-H^3 in vivo

A 245 gm. male rat was treated by intraperitoneal injection with 11.9 μmoles of inositol-2-H^3 (specific activity, 1.43×10^6 c.p.m. per μmole) in two equally divided doses given 1.5 hours apart. The animal was decapitated 1.5 hours after the final dose. Organs were removed and weighed, and 250 mg. portions were treated with trichloroacetic acid. Lipides were extracted as described under "Experimental." The residue insoluble in chloroform-methanol was then dissolved in Hyamine and counted by liquid scintillation techniques (18, 19). The acid-soluble fraction was neutralized with 10 N NaOH and treated with saturated lead acetate. The filtrate was cleared with barium acetate, applied to Dowex 1-formate, and eluted with 2 M formic acid.

Organ	Total radioactivity values		
	Fractions soluble in chloroform-methanol	Fractions insoluble in chloroform-methanol	Total anionic material
	c.p.m.	*c.p.m.*	*c.p.m.*
Liver	103,600	4,435	1,660
Kidney	59,100	9,830	747
Lung	16,580	1,480	493
Testis	12,200	23,400	
Heart	10,560	2,510	
Spleen	9,640	7,050	
Pancreas	9,020	2,840	
Diaphragm	3,970	1,772	
Brain	3,950	63	56

hexokinase. The product was subsequently identified as glucuronic acid, in agreement with the observations of Charalampous (20). Incubation of the kidney residue with glucuronic acid-C^{14} did not indicate that this derivative was converted *in vitro* to inositol or to inositol lipide. The anionic product in liver has not been identified.

Incorporation of Inositol-H^3 into Inositol Lipide—Inositol incorporation into a lipide fraction was previously observed in homogenates of guinea pig kidney (21). Enzymes present in the particulate fraction which sedimented at 7500 × *g* catalyzed this reaction in the presence of Mg^{++} and a cytidine nucleotide. Uridine, adenine, guanine, and inosine nucleotides were ineffective (22). When the kidney residue preparation and inositol-H^3 were incubated in the presence of Mg^{++} and CMP, the most rapid rate of incorporation occurred during the 1st hour of incubation (Fig. 3).

Nucleotide Requirements—Although the requirement for the cytidine moiety was specific, all of the cytidine nucleotides were effective to some extent. CDP-choline was the most effective nucleotide at the concentrations of nucleotide used (1.0×10^{-3} M). The relative efficacy observed in one typical preparation was: CDP-choline, 1.0; CMP, 0.39; CDP, 0.27; CTP, 0.17; cytidine, 0. However, these relative values were found to vary with the type of buffer used. In an experiment in which the residue was suspended in a buffer in which the phosphate was replaced with Tris,[2] pH 7, CDP-choline and CMP stimulated inositol incorporation into lipide to the same extent as they did in the presence of phosphate buffer, whereas CDP and CTP were practically inactive. An increase in phosphate concentration (potassium phosphate buffer, pH 7) from 8×10^{-3} M to 8×10^{-2}

[2] The abbreviation used is: Tris, tris(hydroxymethyl)aminomethane.

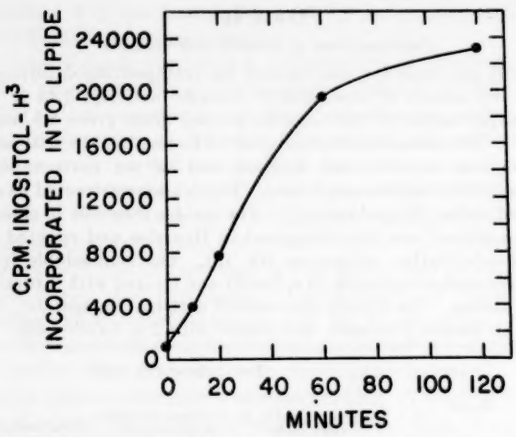

FIG. 3. Time course of inositol incorporation into lipide. The incubation mixtures contained 0.2 ml. of enzyme in the KCl-$MgSO_4$-phosphate buffer to which had been added 10 μmoles of $MgSO_4$, 0.3 μmole of CDP-choline, and 0.2 μmole of inositol-H^3 (2×10^7 c.p.m.), in a total volume of 0.3 ml. The mixtures were incubated for the periods of time indicated at 37° under N_2. Lipides were extracted and counted as described in the text.

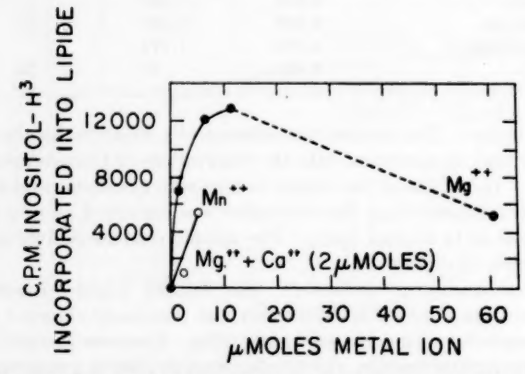

FIG. 4. The effect of metal ions on the incorporation of inositol into lipide. The incubation mixtures contained 0.2 ml. of enzyme in homogenizing buffer from which Mg^{++} had been omitted, 0.2 μmole of inositol-H^3, 0.3 μmole of CDP-choline, and metals as indicated in a total volume of 0.3 ml. Incubations were performed at 37° for 2 hours.

M caused a 40 per cent stimulation of inositol incorporation into lipide in the presence of CDP-choline, but further increases up to 4×10^{-1} M phosphate had no additional effect. In the presence of high levels of phosphate buffer, CDP and CTP were occasionally more effective than CDP-choline or CMP.

Michaelis Constant for Inositol—Experiments in which the inositol concentration was varied in the presence of enzyme, 3.2×10^{-2} M $MgSO_4$, 1.0×10^{-3} M CDP-choline, and 8×10^{-2} M potassium phosphate buffer, pH 7, indicated that the K_m for inositol was 7.2×10^{-4} M in the lipide incorporation system.

Metal Requirements—A requirement for Mg^{++} was established. Mn^{++} was less effective. Mg^{++} was inhibited by low concentrations of Ca^{++} (Fig. 4).

Identification of Inositol Lipide—A pooled sample of chloroform-methanol extracts from experiments *in vitro* with inositol-H^3 was taken to dryness at 40° and chromatographed with diisobutyl ketone-acetic acid-water (40:30:7), as described by Marinetti (23), except that impregnated glass paper was used instead of filter paper. The chromatograms were developed for 3 to 6 hours, dried, and developed with Rhodamine (24). Three distinct spots were obtained which represented the major phospholipides of the enzyme preparation. The choline-containing lipides were identified with phosphomolybdate-$SnCl_2$ spray (25). Comparison of R_F values with authentic lipides indicated that the three spots represented sphingomyelin and possibly lysolecithin (R_F, 0.55), lecithin (R_F, 0.62), and phosphatidyl ethanolamine + phosphatidyl serine (R_F 0.76). About one-half of the recovered radioactivity was eluted from the spot with an R_F value of 0.55 by extraction from the paper with 1 N NH_4OH in methanol. All of the remaining activity was found in the area of paper between the origin and sphingomyelin spot. Chromatography of mixtures of authentic inositol phosphatide and sphingomyelin also resulted in streaking of the inositol phosphatide from the origin to the sphingomyelin spot. With diisobutyl ketone-acetic acid (30:5) on unimpregnated paper (26), authentic inositol phosphatide and sphingomyelin could be separated. When an aliquot of the pooled extract was chromatographed in this system, all the radioactivity recovered was present in the area corresponding to that of inositol phosphatide. Another aliquot was subjected to hydrolysis followed by two-dimensional chromatography of the glycerophosphoryl esters (6). All of the recovered activity was located in a spot corresponding to that obtained with authentic inositol phosphatide.

Effect of Added Lipides—The effect of the addition of various lipides on the incorporation of inositol into inositol phosphatide was examined. As has been observed in studies on lecithin formation (27), the presence of Tween 20 depresses lipide synthesis but it is also very effective for the dispersion of lipide substrates in aqueous systems. The inhibitory action of Tween 20, as well as the effect of various possible lipide acceptors of inositol, is summarized in Table III. The addition of L-α-glycerophosphate, glycerol, or choline was ineffective. In contrast with analogous diglycerides, phosphatidic acids were found to stimulate incorporation of inositol. The experiment with Preparation 3, Table III, was performed after the optimal Mg^{++} and phosphate concentrations had been established. It is clear that the stimulatory effect of the phosphatidic acid is not derived from the release of inorganic phosphate.

The enzymatic activity of kidney preparations was variable. It was observed that the weight of lyophilized preparation obtained from a given number of guinea pig kidneys was a good index of the activity of the inositol incorporation system. A high yield corresponded with high specific activity of the enzyme preparation. The total weight as well as the enzymatic activity per unit weight of the lyophilized residue could be reduced several-fold by fasting the animals for 16 hours before they were killed. The preparations with low enzymatic activity were stimulated by the addition of the boiled residue from an active preparation, but the preparations having high activity were not further stimulated by the addition of the boiled material. In addition, stimulation by phosphatidic acids was more marked in the relatively inactive preparations. These data are consistent with the assumption that the preparations contained quantities of natural lipide acceptors which were affected by

diet, as well as those enzymes which catalyze the incorporation of inositol into lipide.

Formation of Cytidine-Lipide—CMP-H^3 was incubated with the kidney residue preparation in the absence of inositol. Radioactivity was recovered in the chloroform-methanol extract of the trichloroacetic acid-insoluble fraction of the incubation mixture (Table IV). Solvent distribution studies revealed that this radioactive material, soluble in chloroform-methanol, represented a previously undescribed form of cytidine nucleotide (Table V). After alkaline hydrolysis (6) radioactive material was found to be distributed preferentially into the aqueous phase of the two-phase system. The radioactivity in the aqueous phase was readily adsorbed by charcoal from a dilute acetic acid solution (pH, 3 to 4) and was eluted with ethanol-water-1 N NH_4OH (3:3:1). Electrophoresis on paper of the eluate in ammonium formate buffer indicated that radioactivity migrated with CMP and occasionally, but not consistently, there was also a radioactive spot which migrated with CDP-glycerol.

DISCUSSION

Experiments performed *in vitro* indicated that there was a rapid uptake of inositol from the incubation medium by guinea pig kidney tissue slices. Isotopic studies revealed that the cause of this uptake was the conversion of inositol to glucuronic acid as well as the incorporation of inositol into a phosphatide and

TABLE III

Stimulation of incorporation of inositol into lipide by phosphatidic acids

Incubation mixtures contained 0.2 ml. of the enzyme preparation in KCl-$MgSO_4$-phosphate buffer, 0.3 μmole of CDP-choline, and 0.2 μmole of inositol-H^3 (2×10^6 c.p.m.). The lipide esters were added to Tween 20 and water to yield a suspension containing 6 mg. of lipide and 5 mg. of Tween 20 in 0.2 ml., of which 0.03 ml. was added to the incubation mixtures as indicated. For the Tween 20 control incubations, 0.03 ml. of a solution containing 25 mg. of Tween 20 in 1.0 ml. of water was added. Preparation 3 contained in addition 10 μmoles of $MgSO_4$ and 20 μmoles of potassium phosphate buffer, pH 7.0. Total incubation volume in each case was 0.3 ml.

Additions	Radioactivity in lipide
	c.p.m.
Preparation 1	
None	14,440
Choline, 1.0 μmole	15,020
Glycerol, 1.0 μmole	17,060
L-α-glycerophosphate, 1.0 μmole	12,940
Preparation 2	
None	10,720
Tween 20	5,980
Tween 20 + dimyristoyl-L-α-glycerophosphate	17,100
Tween 20 + D-α,β-dimyristin	8,330
Tween 20 + inositol phosphatide	2,250
Preparation 3	
None	61,150
Tween 20	19,880
Tween 20 + dioleoyl-L-α-glycerophosphate	42,750
Tween 20 + D-α,β-diolein	23,820

TABLE IV

Incorporation of CMP-H^3 into lipide extract

Incubation mixtures contained 0.2 ml. of enzyme in the homogenizing buffer to which were added 10 μmoles of $MgSO_4$, 20 μmoles of potassium phosphate buffer, pH 7, and 0.3 μmole of CMP-H^3 (4.2×10^6 c.p.m.) in a total volume of 0.3 ml. Incubation conditions and lipide isolation techniques were those described under "Experimental."

Length of incubation	Radioactivity in chloroform-methanol extract
min.	*c.p.m.*
0	292
30	1047
60	1508
120	1753

TABLE V

Solvent distribution ratios of radioactivity after incubation of CMP-H^3 with kidney enzyme preparation

An incubation mixture 5-fold the quantity of that described in Table IV was incubated for 2 hours, treated with trichloroacetic acid, and extracted with chloroform-methanol, 1:1, as described previously. The extract was shaken for 15 minutes with ½ volume of 0.03 M potassium phosphate buffer, pH 7. The two phases were separated by centrifugation and aliquots of the upper (aqueous) and lower (chloroform) layer were counted. The upper phase was replaced with a new aqueous layer which was obtained from a previously equilibrated solvent system. The mixture was again shaken and centrifuged to obtain a new distribution ratio. This step was repeated and the purified chloroform layer was taken to dryness and subjected to mild alkaline hydrolysis with methanolic NaOH (6). The hydrolysate was passed through a column of Dowex 50, evaporated to dryness, extracted with ether to remove unhydrolyzed material, and again subjected to distribution in the chloroform-methanol-aqueous phosphate buffer mixture.

Step	Radioactivity	
	Total	Distribution ratio of aqueous phase to chloroform phase
	c.p.m.	
1. CMP-H^3*		400:1
2. Chloroform-methanol extract after incubation with CMP-H^3	8812	0.14:1
3. Chloroform-methanol extract after replacement of aqueous phase	8688	0.11:1
4. Chloroform-methanol extract after second replacement of aqueous phase	7452	0.045:1
5. Ether extract after hydrolysis	1338	
6. Hydrolysate	3024	2.7:1
7. Hydrolysate after replacement of chloroform phase	2439	8.8:1

* Solvent distribution ratios were also obtained with nonisotopic compounds by comparison of ultraviolet absorption of the two phases. Ratios of 101:1 to 465:1 were observed for all derivatives examined: cytosine, cytidine, CMP, CDP, CDP-choline, and CDP-glycerol.

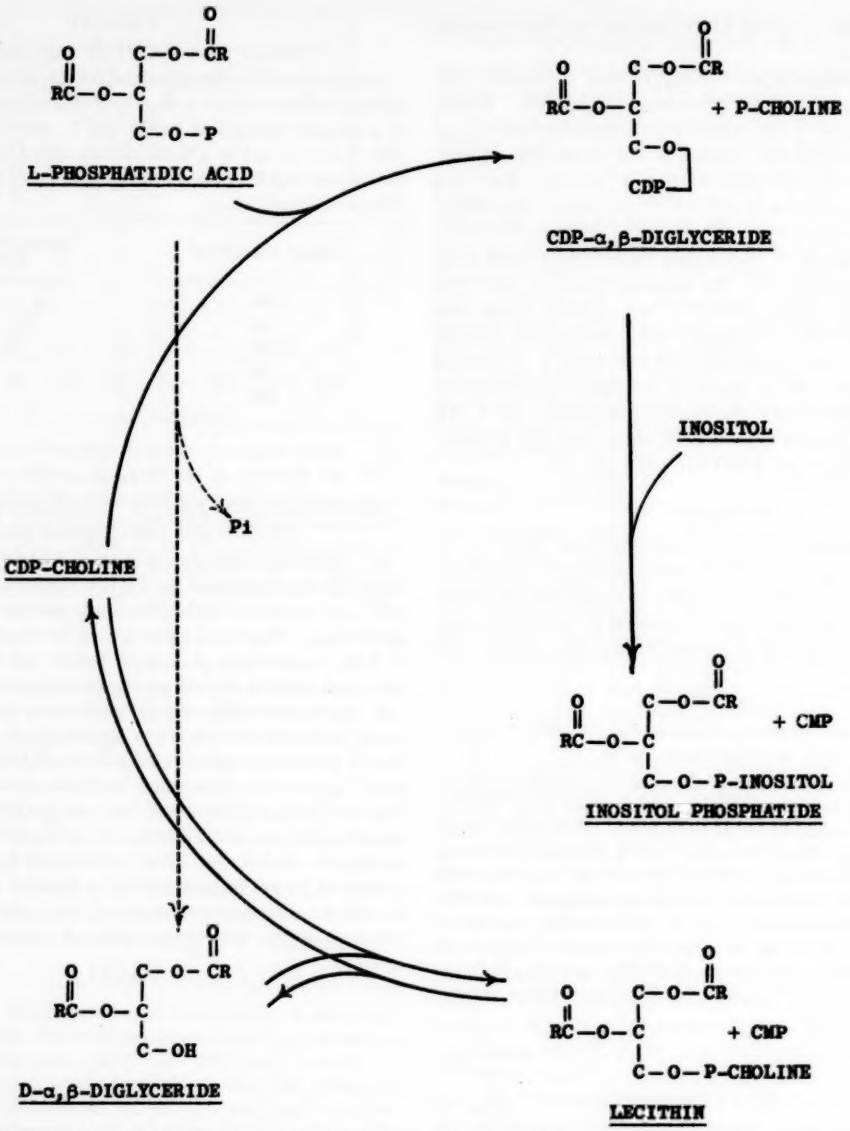

FIG. 5. Proposed relationship of the pathways for inositol phosphatide and lecithin synthesis. CDP-choline may react with either phosphatidic acid to produce a liponucleotide or with diglyceride to form lecithin. The liponucleotide and inositol may then react to form inositol phosphatide.

into other complex substances. The solubility of the radioactive lipides isolated from experiments performed *in vivo* indicated that an inositol lipide is present in liver in addition to the monophosphoinositide which differs qualitatively from the inositol phosphatide of kidney. The trichloroacetic acid-insoluble, chloroform-methanol-insoluble (nonlipide) inositol is probably bound with protein (28) or with nucleic acid (29). The bound nonlipide inositol of brain was found to release inositol[3] by treatment with an acidic lipide solvent as has been observed by Folch (30). There was no apparent correlation between the rate of incorporation of inositol *in vivo* into the lipide and nonlipide fractions of different organs. This finding suggests the existence of independent pathways for the incorporation of inositol into lipide and into other inositol-containing substances.

The studies with enzyme preparations represent another example of the implication of cytidine nucleotides in the formation of phospholipides. A striking characteristic of the cytidine nucleotide requirement for the enzyme system described here is the fact that many cytidine nucleotides are effective. The participation of CDP or CTP requires the presence of inorganic phosphate. Since inositol phosphatide is actively formed in the absence of phosphate, *e.g.* in Tris buffer, and since CDP-choline was more effective than CMP in both Tris and phosphate buffer, it would seem that CDP-choline is the reactant nucleotide in the inositol-incorporating system. The effectiveness of CMP can be explained by its conversion to CDP-choline by diglyceride transferase (31) in the presence of lecithin in the kidney residue.

[3] B. W. Agranoff, unpublished observations.

Chromatographic evidence revealed that the inositol lipide formed was the monophosphatide similar to that isolated from plant sources and from horse liver (5).

In contrast to the enzymes involved in the biosynthesis of choline and phosphatidyl ethanolamine (27), inositol appears to be incorporated into lipide without previous phosphorylation. No evidence for the presence of CDP-inositol was found in experiments with slices or cell-free preparations. Furthermore, the addition of D-α,β-diglycerides did not stimulate the incorporation of inositol as would be expected by analogy with the reaction of CDP-choline (27), whereas phosphatidic acids did stimulate the system.

The participation of phosphatidic acids as lipide acceptors in this system suggested that the primary reaction was a transphosphorylation of CDP-choline and a phosphatidic acid to form CDP-D-α,β-diglyceride as follows: CDP-choline + phosphatidic acid → CDP-D-α,β-diglyceride + P-choline. The CDP-D-α,β-diglyceride then would react with a hydroxyl group of inositol in a pyrophosphorolytic reaction analogous to the reaction of CDP-choline with the free hydroxyl group of the diglyceride in the "glyceride transferase" reaction (27): CDP-D-α,β-diglyceride + inositol → inositol phosphatide + CMP.

Evidence for a cytidine-lipide was demonstrated by the solvent distribution properties of a product obtained after incubation of CMP-H^3 with the enzyme preparation. Hydrolysis yielded CMP and probably CDP-glycerol.[4]

The metabolic role of phosphatidic acid has remained questionable since its identification as the product of an enzymatic reaction between fatty acid acyl-CoA and L-α-glycerophosphate (32). An explanation of the low concentrations of phosphatidic acid in whole tissues (33) is provided by the action of phosphatidic acid phosphatase (34) to yield L-α,β-diglyceride which is then available for formation of lecithin (29) or triglyceride (35, 36). The presence of Mg^{++} inhibits the phosphatase and thus may favor the synthesis of inositol phospholipide rather than the nitrogenous phospholipides or triglyceride via the diglyceride. The rapid incorporation of P^{32} into the inositol lipides and phosphatidic acid, which has been observed *in vitro* (6–9), might be explained by the presence of Mg^{++} at higher concentrations than is found *in vivo*. In addition, the phosphorus of phosphatidic acid and inositol phosphatide are derived from glycerophosphate, whereas the phosphorus of lecithin and phosphatidyl ethanolamine is derived from the phosphorylated base, phosphoryl choline, or phosphoryl ethanolamine. The metabolic interrelationship is summarized in Fig. 5.

SUMMARY

A spectrophotometric method for the determination of inositol is described. Studies *in vitro* indicated active utilization of inositol by kidney tissue. Experiments with tritium-labeled inositol demonstrated the incorporation of inositol into the lipides of all tissues studied.

An enzyme system present in the insoluble residue of guinea pig kidney mitochondria is described which catalyzes the incorporation of inositol into inositol phosphatide in the presence of Mg^{++} and cytidine diphosphate-choline or cytidine-5′-phosphate. The incorporation of inositol was stimulated by the addition of phosphatidic acids, but not by diglycerides. A mechanism is proposed for the synthesis of inositol phosphatide.

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[4] Deacylation of the CDP-diglyceride would yield CDP-glycerol. Attempts at deacylation in methanolic NaOH as well as under milder conditions converted over 80 per cent of authentic CDP-glycerol to CMP. The relative rates of pyrophosphate cleavage and deacylation may account for the varying yields of CDP-glycerol from hydrolysis.

The Biosynthesis of Δ^4-Androstenedione and 17α-Hydroxyprogesterone from Progesterone by Surviving Human Fetal Adrenals

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(Received for publication, April 1, 1958)

In this paper evidence that the human fetal adrenal can convert progesterone into 17α-hydroxyprogesterone and androstenedione is presented. Moreover, for the first time, it has been shown that the portion of the fetal adrenal known as the "fetal zone" can effect these transformations.

In the early months of fetal life the human adrenals grow rapidly and are, by the 3rd month, larger than the kidneys. Thereafter their relative size decreases slowly. At term, however, they are 10 times larger (proportionately by weight) than the adult adrenal. Their size is attributable to a central reticular zone known as the fetal zone which makes up about 80 per cent of the volume of the gland. This zone disappears because of hemorrhage and necrosis in the first few months after birth. Its function is not known, although its peculiar developmental course has excited much speculation. Experimental work has been difficult, because no readily available laboratory animal has a definitely homologous structure; the fetal zone occurs only in primates. Early attempts to establish a hormonal function for fetal adrenals by extraction and bioassay failed uniformly. More recently chemical methods have been employed. Bloch *et al.* (1, 2) have analyzed extracts of adrenal tissue from human fetuses and have detected three C_{19}-17-ketosteroids which, because of the minute amounts present, were identified only tentatively by paper chromatographic methods. As has already been pointed out (3), the detection of ketosteroids in adrenal extracts does not necessarily prove that biosynthesis occurred in that gland. Lanman and Silverman (3) provided evidence that human adrenals of newborn infants could, during incubation, convert progesterone into hydrocortisone, corticosterone, and possibly also Compound S (11-desoxy-17α-hydroxycorticosterone). None of the previous studies demonstrated whether the fetal zone, as distinct from the whole adrenal, is capable of carrying out biochemical transformations for hormone synthesis.

The ready availability of normal human fetal tissue from legal abortions in Sweden suggested the possibility of further experimental work designed to establish the biogenetic capacity of the fetal zone as distinct from the adult type of tissue.

EXPERIMENTAL

The design of this study involved the dissection of fetal adrenals obtained as soon as possible (within 15 minutes) after abortion into two fractions. One of these consisted almost entirely of the fetal zone, and the other, the outer portion, consisted mainly of the adult zone but was unavoidably contaminated with fetal zone. Each fraction, after appropriate preparation, was incubated with progesterone-4-C^{14} together with two nonradioactive "trapping agents," 17α-hydroxyprogesterone and Δ^4-androstene-3,17-dione. After incubation the trapping agents were isolated and carefully purified to constant specific activity. To insure as far as possible radiochemical homogeneity, various chemical transformations were carried out. The retention of radioactivity by the trapping agents after the termination of these procedures was accepted as proof that the precursor, progesterone, had been converted by the action of the enzyme systems present in the tissue under investigation into the added carriers.

Determination of Radioactivity—Aliquots of samples to be assayed were plated directly on aluminum planchets (1.25 inches in diameter and $\frac{3}{32}$ inch in depth) and counted in a Nuclear Chicago D-46 windowless gas flow counter, operated in the Geiger region. All counts were determined at infinite thinness with not more than 250 μg. of material plated on each planchet. Sufficient counts were accumulated to give a standard error of no more than 2 per cent (4). The specific activities recorded below are all expressed as counts per minute per mg. Radioautographs of the paper chromatograms were prepared with the use of "no screen" Kodak x-ray films according to the procedure described by Taurog *et al.* (5). After exposure for 2 to 7 days, the developed films were compared with the ultraviolet-absorbing zones on the paper.

Melting Points and Infrared Spectra—All melting points were taken on a Kofler block and corrected. Infrared spectra were determined in a model 21C Perkin-Elmer double beam spectrophotometer, either as KBr disks or in CS_2 solution.

Preparation and Incubation of Adrenal Glands—Normal human fetal adrenal glands were obtained in Sweden from legal abortions for nonmedical indications. The glands were frozen within 15 minutes of delivery, and while frozen they were sliced into layers 2 or 3 mm. thick. The outer portion of each slice was cut away with a razor blade. This portion contained the adult type of cortex (zona glomerulosa and zona fasciculata) as well as a thick layer of fetal zone tissue. This portion will be referred to as the mixed tissue fraction. The remaining inner portion

contained almost pure fetal zone tissue. Histological section revealed that good separation was achieved, although occasional fragments of the adult type of cortex were found. This portion will be referred to as the fetal zone fraction. There is relatively little medullary tissue in the human fetal adrenal. Subsequently each fraction was treated separately but identically.

Within 2 or 3 hours after freezing, a 10 per cent (weight per volume) homogenate was made with a Potter-Elvehjem type homogenizer and cold phosphate buffer (pH 7.4, 0.02 M). Sodium fumarate, nicotinamide, and magnesium chloride were added to final concentrations of 0.04 M, 0.04 M, and 0.007 M, respectively. Radioactive progesterone and nonradioactive carriers androstenedione and 17α-hydroxyprogesterone were added, and the homogenates were incubated at 37° in air. After 10 minutes adenosine triphosphate (disodium salt) and diphosphopyridine nucleotide (Pabst Laboratories) were added to a final concentration of 0.004 M for each. Incubations were continued for 2 hours.

A total of 26 pairs of adrenal glands was studied. Two-thirds of the fetuses from which these glands were obtained were of 14 to 18 weeks of age, and the remainder were between 10 and 22 weeks. Although collected over a period of 1 month, the adrenal glands were incubated as soon as possible after abortion. Thus 15 separate experiments, each consisting of two incubations (one with the mixed tissue fraction and the other with the fetal zone fraction) were carried out. The total amount of tissue incubated was 7.27 gm. of the mixed tissue fraction and 7.49 gm. of the fetal zone fraction. Each fraction had been incubated with 3.58 mg. of progesterone-4-C^{14} (7.2×10^6 c.p.m. per mg. (Volk) and 5 mg. each of carrier, unlabeled androstenedione, and 17α-hydroxyprogesterone. The three steroids were dissolved in 3 ml. of propylene glycol and appropriately apportioned between the incubations. After incubation, acetone extracts of each were stored and combined with other similar extracts.

Extraction and Preliminary Fractionation—After incubation the homogenates were added to 10 volumes of acetone with stirring, and the mixture was stored in a cold temperature overnight. The precipitated proteins were filtered and washed with cold acetone, and the combined acetone filtrates were evaporated to dryness in a vacuum. At this point the acetone residues from the individual experiments were pooled (Stage A, Table I). These residues were then defatted by means of a 6 funnel countercurrent distribution between 90 per cent methanol and hexane. The 90 per cent methanol phases were combined and taken to dryness in a vacuum (Stage B, Table I). The residue obtained from the outer zone experiments was dissolved in toluene, and the toluene solution was partitioned into a neutral fraction (Stage C) and a phenolic fraction (Stage D) by the procedure of Engel *et al.* (6). To avoid troublesome emulsions which resulted from this procedure, the residue from the fetal zone incubation was dissolved in chloroform. The phenolic and neutral fractions were then easily obtained by the above procedure (6). The distribution of the radioactivity among the various fractions is shown in Table I. The nature of the radioactive metabolites in the phenolic fraction was not investigated further.

The neutral extracts (Table I) of fetal and outer zones were chromatographed separately on silica gel; for the fetal zone extract, 18 gm. of silica gel in a 10.5 × 1.8 mm. column were used. The extracts were put on the adsorbent with ligroin, and elution was effected by 25 ml. portions of solvents of increasing polarity. From the distribution of the radioactivity in the eluates, appropriate fractions were combined into four main portions named A, B, C, and D. The weights and radioactivity

TABLE I

Distribution of radioactivity in fractionation procedure

Stage	Fraction	Fetal zone fraction	Mixed tissue fraction
		c.p.m. × 10^6	*c.p.m.* × 10^6
A	Acetone residue	20.95	21.97
B	90% Methanol residue	21.83	19.13
C	Neutral	19.36	19.91
D	Phenolic	0.20	0.27

TABLE II

Chromatography of extract of mixed tissue fraction on silica gel

Elution sequence	Volume	c.p.m. × 10^6	Weight
	ml.		*mg.*
Fraction A			
Ligroin B-benzene (1:1)	5 × 25		
Benzene	5 × 25	2.72	135
Ethyl acetate-benzene (2:98)	2 × 25		
Fraction B			
Ethyl acetate-benzene (2:98)	6 × 25		
Ethyl acetate-benzene (1:9)	22 × 25	5.49	15
Ethyl acetate-benzene (1:3)	8 × 25		
Fraction C			
Ethyl acetate-benzene (1:1)	12 × 25		
Ethyl acetate	4 × 25	8.10	8
Ethanol-ethyl acetate (1:9)	8 × 25		
Fraction D			
Ethanol-ethyl acetate (1:1)	200	0.49	8

TABLE III

Chromatography of extract of fetal zone on silica gel

Elution sequence	Volume	c.p.m. × 10^6	Weight
	ml.		*mg.*
Fraction A			
Ligroin B-benzene (1:1)	4 × 35	1.76	93
Benzene	2 × 35		
Fraction B			
Ethyl acetate-benzene (1:9)	6 × 35	4.02	41
Ethyl acetate-benzene (2:8)	8 × 35		
Fraction C			
Ethyl acetate-benzene (1:1)	10 × 35		
Ethyl acetate-benzene (8:2)	11 × 35	9.92	27
Ethyl acetate	7 × 35		
Ethanol-ethyl acetate (1:9)	8 × 35		
Fraction D			
Ethanol-ethyl acetate (1:1)	270	2.44	52
Ethanol	200		

of these are given in Tables II and III. Fraction A of both zones was not investigated further since progesterone itself is found in these eluates. Since deoxycorticosterone is eluted from silica gel with benzene-ethyl acetate (1:1), Fractions B and C were processed separately. This division simplified subsequent chromatographic separations of Fractions B and C. Fraction D was not further investigated.

The paper chromatographic systems described by Burton *et al.* (7) and Savard (8) were adopted for further resolution. The systems used were: ligroin C-propylene glycol (System A), benzene:cyclohexane-propylene glycol (System B), and toluene-propylene glycol (System C). The chromatograms were analyzed for ultraviolet-absorbing material by means of an ultraviolet lamp and for radioactivity by means of radioautography. The material present in each absorbing zone was eluted from the paper by cutting the appropriate areas into small fragments and soaking these in methanol overnight. The residues that remained after the filtered methanol solutions were evaporated to dryness in a vacuum at 40° were partitioned between ethyl acetate and water to remove traces of the stationary phase which were usually present at this point. The ethyl acetate-extracted material was then counted. In general, approximately 90 per cent of the radioactivity applied to the paper could be accounted for after elution.

RESULTS

Isolation and Radiochemical Characterization of Carriers

1. Incubated Mixed Tissue Fraction—Fraction B (Table II) was chromatographed (paper chromatography) in System A for 10¼ hours (Chromatogram I). During this time the progesterone standard ran off the paper. Four main radioactive and ultraviolet-positive zones were resolved on the chromatogram. One moved at a rate corresponding to that of 17α-hydroxyprogesterone, and another had the mobility corresponding to that of androstenedione. The other two zones were not investigated further.

The androstenedione zone (820,000 c.p.m.) was purified by chromatography in System A for 15 hours (Chromatogram Ia). One radioactive zone, which moved with the mobility of androstenedione, was detected. Elution from the paper resulted in a crystalline sample, the further purification of which is described below.

The radioactive material present in the zone assumed to be 17α-hydroxyprogesterone (Chromatogram I) was chromatographed in System B for 12 hours (Chromatogram Ib). Four zones were detected, only one of which had the mobility of 17α-hydroxyprogesterone. This zone was counted at 426,000 c.p.m.

When Fraction C (Table II) was initially chromatographed in System C for 16 hours (Chromatogram II), the presence of seven distinct radioactive and ultraviolet-positive areas was revealed. Efforts to identify these compounds, only one of which is less polar than corticosterone, are in progress. The runoff from Chromatogram II was chromatographed in System C for 4 hours (Chromatogram IIa) and yielded four distinct radioactive areas, one of which corresponded in mobility to 17α-hydroxyprogesterone. This was chromatographed in System B for 8 hours (Chromatogram IIb), and a single zone (290,000 c.p.m.) with the mobility of 17α-hydroxyprogesterone was recovered. This was combined with the fraction from Chromatogram Ib (426,000 c.p.m.) which migrated at the same rate and was further purified as described below.

The crystalline material isolated from Chromatogram Ia weighed 3.8 mg. and counted 769,000 c.p.m. It was further purified by chromatography on alumina (Harshaw Chemical Company). Elution by benzene and benzene containing 3 per cent ether yielded 2 mg. of crystalline material (562,000 c.p.m.) which, upon recrystallization from ether, melted at 170–172°. This material was identified by infrared analysis as androstenedione. Its specific activity was 247,000 which indicated a minimal conversion from progesterone of 4.3 per cent.[1]

The crystals and the mother liquor were then recombined and mixed with 100 mg. of carrier androstenedione. The mixture was recrystallized from acetone-ether to give a product of specific activity of 4780. The second crop from the mother liquor gave a sample with a specific activity of 5150. A second recrystallization of the material (specific activity, 4780) from ether-ligroin yielded androstenedione with a specific activity of 5080.

In order to establish further the radiochemical homogeneity of androstenedione, 40 mg. of the sample with a specific activity of 5080 were converted into testosterone by the procedure of Bernstein *et al.* (9). This procedure involved formation of the enol ether of the α,β-unsaturated ketone, reduction of the 17-ketone with $LiAlH_4$, and regeneration of the α,β-unsaturated ketone by hydrolysis. In this way 25 mg. of a crystalline residue were obtained which upon purification by chromatography on alumina, yielded 12 mg. of testosterone, as evidenced by the melting point (156–157°, from acetone-water-ligroin B) and infrared spectrum (in CS_2). Its specific activity was 5360.

The combined 17α-hydroxyprogesterone fractions from Chromatograms Ib and IIb (total counts were 426,000 and 290,000 c.p.m.) were submitted to a preliminary chromatogram on silica gel which resulted in a semicrystalline product containing 620 μg. of α,β-unsaturated ketone as estimated by its absorption at 240 mμ. It counted at 635,000 c.p.m. This fraction was diluted with 100 mg. of carrier 17α-hydroxyprogesterone, and the mixture was recrystallized twice from acetone. The sample from the first crystallization had a specific activity of 3300 and from the second recrystallization, a specific activity of 3125. The mother liquors from each crystallization deposited second crops; the specific activity of the first product was 3660 and that of the second sample, 3110.

Approximately one-half of the radioactivity originally associated with the 17α-hydroxyprogesterone zones of Chromatograms Ia and IIb did not follow the crystalline carrier and was actually associated with contaminants which were removed in the mother liquors. Since testosterone has mobility properties similar to those of 17α-hydroxyprogesterone in the chromatographic systems used and would yield androstenedione upon oxidation (see below), an effort was made to determine whether a radioactive impurity in the mother liquor was testosterone. 20 mg. of testosterone were added to the mother liquor, the mixture was benzoylated, and the products were separated by chromatography on alumina. Crystalline testosterone benzoate was recovered without radioactivity.

[1] The minimal percentage of conversion from progesterone is equal to the specific activity of the isolated androstenedione × total weight of carrier added ÷ c.p.m. of progesterone initially used, *e.g.* 247,000 × 5.0 × 100 ÷ 28.5 × 10^6 = 4.3 per cent.

To insure the radiochemical homogeneity of the isolated 17α-hydroxyprogesterone, 25 mg. of the sample (specific activity, 3125; see above) were oxidized to androstenedione by the procedure of von Euw and Reichstein (10). The oxidation product, weighing 24 mg., was purified by chromatography on alumina (Harshaw Chemical Company). A total of 12 mg. of pure material was eluted with ligroin-benzene, 40 per cent, and ligroin-benzene, 60 per cent, which upon crystallization from ligroin B-ether gave needles with a melting point of 144–145°[2] and an infrared spectrum identical to that of Δ⁴-androstene-3,17-dione. Its specific activity was 3668.

2. Incubated Fetal Zone—The isolation and radiochemical characterization of the carriers Δ⁴-androstenedione and 17α-hydroxyprogesterone from the incubation of the fetal zone were carried out in essentially the same manner as that applied to the incubates of the mixed tissue fraction. Fraction B (Table III) was chromatographed in System A for 15 hours (Chromatogram III). The androstenedione zone, after an additional chromatogram in System A and recrystallization from ligroin A-ether, gave the crystalline diketone. Identified by infrared analysis, it melted at 170–172° and weighed 1.3 mg. Its specific activity was 272,000 which indicated a minimal conversion from progesterone of 4.8 per cent.[1] As before, the crystalline material was diluted with 100 mg. of carrier androstenedione and recrystallized to a constant specific activity of 4030. Conversion of an aliquot of this to testosterone by the procedure described above afforded a product of specific activity of 3650.

The 17α-hydroxyprogesterone zone from Chromatogram III was combined with a similar area obtained from Fraction C (Table III) after two chromatograms in System B. The combined fractions containing 580,000 counts gave, after chromatography on silica gel and on paper in System B, a zone containing 154 μg. (estimated by its absorption at 240 mμ) of α,β-unsaturated ketone and counting 200,000 c.p.m. It was diluted with 100 mg. of carrier 17α-hydroxyprogesterone and recrystallized to a constant specific activity of 288. An aliquot of this product was oxidized to androstenedione of specific activity of 274. As before, most of the radioactivity associated with the 154 μg. of unsaturated ketone did not follow the crystalline carrier. Again, testosterone was sought in the mother liquors by addition of carrier and benzoylation. The recovered testosterone benzoate, however, was devoid of radioactivity.

DISCUSSION

In this investigation we have attempted, by means of the trapping technique, to demonstrate that the fetal adrenal has the capacity to convert progesterone to 17α-hydroxyprogesterone and to androstenedione. In the case of androstenedione, it was possible to isolate the carrier from the incubation mixture in crystalline form and to measure its specific activity accurately. The constancy of this specific activity after recrystallization, dilution with additional carrier, and recrystallization of the diluted mixture, and conversion to testosterone is considered as evidence of radiochemical purity. Based on the specific activity of the isolated trap calculation of a minimal percentage of conversion of androstenedione from progesterone has been possible. On the other hand, the 17α-hydroxyprogesterone isolated from the incubation mixture was obtained in microgram quantities and was not crystalline. Although spectrophotometric estimation of the amount present was possible, no accurate value for the specific activity could be calculated, since it was found that most of the radioactivity in the fractions thought to be 17α-hydroxyprogesterone was removed in the subsequent purification steps. This finding illustrates the magnitude of the inaccuracy that was possible had it been assumed that the radioactivity found in the single spot migrating at a rate exactly equal to that of 17α-hydroxyprogesterone was associated only with that compound.

In the work-up of the mixed tissue zone two fractions were considered to contain 17α-hydroxyprogesterone. Each had migrated with the correct mobility in two chromatographic systems. The total counts in these fractions from chromatograms Ib and IIb was 716,000 c.p.m. Additional chromatography on silica gel gave 620 μg. of semicrystalline material containing 635,000 c.p.m. Had the specific activity of this sample been calculated from these data, a value twice as high as that ultimately found after further purification would have been recorded. A similar analysis of the radioactivity of the 17α-hydroxyprogesterone isolated from the fetal zone reveals that a *7-fold* difference exists between the specific activity calculated after two chromatographic separations and that ultimately obtained after conversion to androstenedione.

We believe that these studies establish the fetal zone of the human adrenal gland as tissue capable of carrying out biochemical transformations required for hormone synthesis. Previous studies based on gland extracts or incubation of undissected glands have failed to demonstrate the function of that particular tissue which makes the primate gland unique.

17α-hydroxyprogesterone was selected as a carrier because it occupies a unique position in the pathways of steroid biosynthesis. It may be considered a branching point in the path, since it can serve as a precursor of the C_{21} corticosteroid, hydrocortisone, on one hand and of the C_{19} steroid, androstenedione (or testosterone), on the other. Its formation from progesterone demonstrates the presence of a 17α-hydroxylase in fetal adrenal tissue. Androstenedione was chosen as a carrier because its formation from progesterone would indeed prove that an androgenic substance could be made from the appropriate precursor by fetal adrenals. That these adrenals possess an androgenic function has been suspected for many years. In 1933 Broster and Vines (12) demonstrated a rather nonspecific staining similarity between the fetal zone and known androgenic tissue. Grollman (13), in 1936, named the fetal zone the "androgenic zone" but was unsuccessful in demonstrating androgenic activity in glandular extracts. More recently, chemical assays of 17-ketosteroids in the blood and urine of newborn infants have suggested increased androgenic activity, but the site of origin of the androgens remained unknown. Moreover, the chemical estimation, which has usually ended with the Zimmermann assay, is particularly subject to interference from 20-ketosteroids such as those which may be present in large amounts during pregnancy. In addition, the Zimmermann reaction could not distinguish between 17-ketosteroids derived from the fetal adrenal androgen and those 11-oxy-17-ketosteroids derived from 11-oxy-corticosteroids such as hydrocortisone nor from those 17-ketosteroids arising from the maternal placenta or adrenals. The recent isolation of androstanediol from the urine of newborn

[2] Androstenedione has two melting points; if recrystallized from acetone it melts at 173–174°, but if recrystallized from ligroin or ether-ligroin it melts at 142–144° (11).

infants (14) emphasizes the difficulty of estimating androgen production by colorimetric determinations of the 17-ketosteroids.

The presence of numerous other as yet unidentified, more polar, steroid-like compounds suggests that the biosynthetic capacities of the fetal adrenals are not confined to the formation of 17α-hydroxyprogesterone and androstenedione. It may be recalled that Lanman and Silverman (3) have already provided evidence for the biogenesis of cortical steroids (Compounds B, F and S) during incubation of sliced adrenals from newborn infants. The identification of the more polar compounds detected in the present study will be reported in a subsequent paper.

Thus it appears that, for the conversion of progesterone to androstenedione and 17α-hydroxyprogesterone, both zones of the fetal adrenal cortex use the same biochemical mechanisms as do adult adrenals (15), the ovaries (16), and the testes (17–19). This observation serves to support the generalization expressed by Samuels (20) that all steroid-producing endocrine glands possess the enzyme systems necessary to convert cholesterol into all the known steroid hormones. The glands differ from each other in the relative proportions of the various enzymes they possess. These and probably other factors determine which end product is produced in the largest amounts. With this concept it becomes possible to understand how, in pathological circumstances, the adrenals or the ovaries can secrete androgens and the testes, estrogens. The data of this study do not bear upon the hypothesis proposed by Dorfman (21) that the C_{19} steroid, androstenedione, also arises via other pathways which do not involve progesterone and 17α-hydroxyprogesterone.

SUMMARY

Human fetal adrenals have been partitioned by dissection into two fractions, one consisting of "fetal zone" and the other consisting mainly of adult tissue contaminated with fetal tissue. Homogenates of each fraction have been incubated with progesterone-4-C^{14} together with two nonradioactive trapping agents. Isolation of the carriers in radioactive form after extensive purification demonstrated that in these tissue fractions progesterone gives rise to 17α-hydroxyprogesterone and androstenedione. Thus it has been established that the fetal adrenal, and particularly that portion of the fetal adrenal known as the fetal zone, is capable of carrying out some of the steroidogenic transformations usually associated with the human adult adrenal gland. The necessity for the establishment of radiochemical homogeneity of the isolated trapping agents has been indicated.

Acknowledgment—The authors gratefully acknowledge the support given them by The Association for the Aid of Crippled Children and by research grants (A-1437) from the National Institutes of Health, United States Public Health Service and (G-1268) from The Division of Biological and Medical Sciences of the National Science Foundation.

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The Biochemical Transformation of Cholestenone to Cholesterol*

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(Received for publication, April 24, 1958)

The biochemical conversion of a Δ^4-3-ketosteroid to a Δ^5-3-hydroxysteroid has, to our knowledge, been reported only twice. In 1942 Marker *et al.* showed that 4-dehydrotigogen-3-one was, in part, transformed to diosgenin, the corresponding Δ^5-3-hydroxy compound, when fed to dogs (1); and recently, in an abstract, Vishniac and Nielson have reported that cholestenone was reduced to an unsaturated 3β-hydroxysteroid by *Labyrinthula vitellina* (2). It was believed that such a transformation could be effected by the microorganisms in feces and that this could be demonstrated by the tracer technique. These conditions have previously been used to study the mechanism of conversion of cholesterol to coprostanol (3).

It was found that when radiochemically pure Δ^4-cholestene-3-one-4-C^{14}[1] was incubated with a suspension of human feces, a small but significant amount was converted to cholesterol. Further, cholestenone was neither present in measurable amounts in feces nor did it accumulate during the incubation period. Other transformation products obtained from Δ^4-cholestenone were coprostanol, (usually the principal metabolite), coprostanone, and cholestanol.

EXPERIMENTAL[2]

Cholesterol-4-C^{14} and cholestenone-4-C^{14} were purchased from the Beta Laboratories (Philadelphia, Pennsylvania). The purity of the compounds was measured as follows:

1. Cholesterol-4-C^{14}.—To 43.7 mg. of nonradioactive cholesterol dissolved in petroleum ether was added 0.097 mg. of cholesterol-4-C^{14}; after removal of the solvent the cholesterol had a specific activity of 1450 c.p.m. per mg. The specific activity was unchanged after chromatography on alumina, and further recrystallization from acetone did not alter the initial specific activity.

2. Cholestenone-4-C^{14}.—The purchased product was sublimed in high vacuum before use. In order to establish the purity, 0.372 mg. of the sublimate was added to 109.7 mg. of carrier cholestenone in petroleum ether, and this material had a specific activity of 4280 c.p.m. per mg. After chromatography on alumina the product had 4000 c.p.m., and after recrystallization from methanol the specific activity was 4100 c.p.m. per mg.

1 gm. of carrier cholesterol was added to 1.12 mg. of sublimed cholestenone-4-C^{14} (1.27×10^6 c.p.m. per mg.). The mixture was separated into ketonic and nonketonic fractions by two treatments with Girard's Reagent T. One recrystallization of the nonketonic portion from acetone afforded cholesterol which was nonradioactive when measured by the counting method used throughout the study.

Δ^5-Cholestene-3-one-4-C^{14}.—This substance was prepared from cholesterol-4-C^{14} (12,000 c.p.m. per mg.) by the method of Butenandt and Schmidt-Thomé (4). The product melted at 117-121°, and the infrared spectrum in carbon disulfide agreed with that of an authentic sample; from $E^{1\%}_{1\,cm.} = 45.1$ at 240 mμ in ethanol the product contained 10 per cent of the Δ^4-isomer.

Transformation of Cholestenone-4-C^{14} by Feces

The amounts and the total radioactivity of the compounds added to the incubation medium are recorded in Table I. A fresh stool collection from the same subject was used for each experiment. In Experiment I approximately 40 per cent of the stool was incubated with labeled cholesterol (Experiment IB), and in Experiment III the sample was divided into two equal parts before addition of the labeled compound. The conditions for incubation (2 days at 37°) and isolation of the nonsaponifiable fractions were identical with the procedure reported earlier (3). Table I also shows the weights of the nonsaponifiable and the acidic fractions obtained after each experiment as well as the amount of radioactivity which was recovered in each fraction. The nonsaponifiable portions were chromatographed on alumina columns, and four principal fractions were obtained. In order of elution these were: (*a*) coprostanone, (*b*) coprostanol, (*c*) cholesterol, and (*d*) the more polar material after elution of cholesterol. Cholestenone was intermediate between coprostanol and cholesterol.

Cholesterol and coprostanol were rigorously purified; criteria for purity were melting point, infrared spectrum in carbon disulfide solution between 1400 and 650 cm.$^{-1}$, and the constancy of specific activity through several purification steps (Table II). Cholestenone was not detected in the eluate except in Experiment III in which approximately 200 mg. of ketone were added. In Experiments IA and II its presence was indicated by the comparatively large amount of radioactivity in the late coprostanol or early cholesterol fractions. Carrier cholestenone was added to this material, and the ketone was reisolated and purified to constant specific activity. Coprostanone and choles-

* This investigation was supported in part by a grant from the American Cancer Society and a research grant (No. CY-3207) from the National Cancer Institute, National Institutes of Health, United States Public Health Service. A preliminary report of this work was presented at the 133rd annual meeting of the American Chemical Society in San Francisco, California, in April, 1958.

[1] The abbreviation used is: cholestenone-4-C^{14} for Δ^4-cholestene-3-one-4-C^{14}.

[2] All melting points are corrected.

TABLE I

Addition and recovery of radioactivity

Experiment	Additions to medium	Added radioactivity (c.p.m. × 10^{-6})	Recovery of fractions			
			Nonsaponifiable		Acid	
			mg.*	%†	mg.	%
IA	0.745 mg. of cholestenone-4-C^{14} + 110 mg. of cholesterol	0.943	2931	93	13128	0‡
IB	110 mg. of cholesterol-4-C^{14}	0.571	1826	97	7768	0‡
II	2.6 mg. of cholestenone-4-C^{14} + 200 mg. cholesterol	3.00	2339	70	5219	17
IIIA	212 mg. of Δ^5-cholestenone-4-C^{14}	2.12	1070	66	3881	18
IIIB	207 mg. of cholestenone-4-C^{14}	2.00	1010	76	3813	17
IV	2.22 mg. of cholestenone-4-C^{14} + 226 mg. of cholesterol	5.36	4826	82		

* The weights of the nonsaponifiable and acid fractions differed in each stool collection. This is due to the various factors (dietary, microbial, and intestinal) which affect the quantity and composition of feces.

† Per cent of total radioactivity.

‡ No significant radioactivity was detected in these fractions.

tanol, present in small amounts, were purified to the extent permitted by the quantity available.

Coprostanone, Experiments IA and III—The coprostanone fractions were combined and counted.

Experiment II—The fractions were combined and treated with Girard's Reagent T; the ketonic portion was chromatographed on alumina.

Coprostanol, Experiments I, II, and III—Crude material was recrystallized twice from methanol, acetylated, and treated with perbenzoic acid (3). The product was chromatographed on alumina, and coprostanyl acetate was recrystallized twice from methanol.

Experiment IV—The combined coprostanol fraction was recrystallized from methanol and adjudged pure by its melting point and by chromatography on paper in the phenyl-Cellosolve-pentane system (5).

Cholestenone, Experiment II—The eluates immediately preceding the main cholesterol fraction were highly radioactive (2440 c.p.m. per mg.). To 15.9 mg. of this material were added 101.7 mg. of carrier cholestenone, and the mixture was chromatographed on alumina. The cholestenone was treated with Girard's Reagent T, and the ketonic fraction (330 c.p.m. per mg. or 2440 c.p.m. per mg. after correcting for dilution) was sublimed. The sublimate was recrystallized twice from methanol (1960 and 2070 c.p.m. per mg., respectively).

Experiments IIIA and IIIB[3]—The cholestenone which was eluted from the column was contaminated with coprostanol, and the mixture was separated by Girard's Reagent T. In Experiment IIIA the ketonic portion had a specific activity of 2340 c.p.m. per mg., and the nonketonic fraction had 246 c.p.m. per mg.; the corresponding values in Experiment IIIB were 3140 c.p.m. per mg. for the ketonic portion and 610 c.p.m. per mg. for the nonketonic portion. The ultraviolet spectrum in ethanol showed that the ketonic fraction contained 25 per cent of the α,β-unsaturated ketone in A ($E^{1\%}_{1cm.}$ = 102 at 240 mμ) and 35 per cent in B ($E^{1\%}_{1cm.}$ = 140 at 240 mμ). This fraction was chromatographed on alumina.[4] Infrared spectrometry showed that coprostanol was the principal contaminant of the eluates. The ultraviolet absorption in ethanol at 240 mμ measured the amount of unsaturated ketone in the fractions; these results and the corresponding specific activities are shown in Table III.

Cholesterol

Experiment IA—Cholesterol from the chromatogram was contaminated with cholestenone which was removed by a second chromatogram on alumina. The cholesterol fraction was treated with Girard's Reagent T, and the nonketonic fraction was recrystallized twice from acetone (m.p. 115–124°). The infrared spectrum indicated that the product was cholesterol mixed with cholestanol.

Experiment IB—The combined cholesterol fraction was acetylated with acetic anhydride in pyridine and oxidized with perbenzoic acid. The oxidation product was chromatographed on alumina. The eluates with the major portion of the radioactivity had an infrared spectrum identical with that of 3β-acetoxycholestane-5,6-epoxide. This compound was recrystallized from methanol with no change in radioactivity.

Experiments II and III—The cholesterol was pruified by formation of cholesterol dibromide and regeneration of the sterol by refluxing the dibromide with sodium iodide in benzene. Cholesterol was acetylated, and the product was chromatographed on alumina. Cholesteryl acetate was recrystallized from acetone, saponified, and mixed with an equal portion of carrier cholestenone. Chromatography of the mixture on alumina afforded cholesterol with unchanged specific activity after two crystallizations from methanol. The cholestenone obtained from this chromatogram was devoid of radioactivity.

Experiment IV—Table IV is a summary of the purification steps and the accompanying changes in specific activity of the cholesterol. The major portion of the cholesterol fraction was subjected to two "washout" procedures, the first with an equal amount of coprostanol and the second with an equal weight of cholestenone. Two recrystallizations of the cholesterol fraction from acetone and a final crystallization from methanol afforded a product which melted at 120–126° and was a mixture of cholesterol and cholestanol from the infrared spectrum. Crystals and mother liquors were combined and treated with hydrogen peroxide according to the method of Fieser and Rajogopalan (6). The hydroxylated mixture was chromato-

[3] Δ^5-Cholestenone is isomerized to the conjugated ketone by saponification of the incubation mixture; therefore, Δ^4-cholestenone was isolated in both experiments.

[4] Cholestenone was not easily separated from coprostanol with Girard's Reagent T. The ketonic portion always contained some nonketonic material. Upon chromatography of this simple mixture, cholestenone was eluted in the early coprostanol fractions. This differed from the order of elution when the total nonsaponifiable fraction was chromatographed.

TABLE II

Transformation of cholesterol-4-C^{14} and cholestenone-4-C^{14} by feces

Experiment	From nonsaponifiable fraction															Other products§
	Coprostanone			Coprostanol			Cholestenone			Cholesterol			Cholestanol			
	% N.S.*	c.p.m./mg.	% conversion†	% N.S.	c.p.m./mg.	% conversion	% N.S.	c.p.m./mg.	% conversion	% N.S.	c.p.m./mg.	% conversion	% N.S.	c.p.m./mg.	% conversion	% conversion
IA	10.5	340	11.0	58.5	306	56			14.3‡	3.5	62	0.7				8.0
IB	7.7	260	6.4	57.0	258	47				5.1	1250	20				9.6
II	7.6	910	5.4	50.2	930	36			1.7‡	11.3	146	1.3	0.3	154	0.04	4.7
IIIA	9.3	310	1.5	36.2	136	2.5	6.4	9300	30	10.8	126	0.7		33	<0.01	16.2
IIIB	11.6		4.9	39.1	295	5.8	8.0	8900	36	9.7	86	0.4		55	<0.01	9.0
IV				44.3	1580	63				5.6	262	1.3				

* *N.S.*, nonsaponifiable.

† The percentage of conversion is based on total radioactivity added to feces.

‡ Calculated from the specific activity of the fractions containing cholestenone (IA = 2500 c.p.m. per mg., II = 2070 c.p.m. per mg.). The radioactivity was shown to be almost completely associated with cholestenone by reverse isotopic dilution.

§ Combined material which was eluted from the chromatogram after the cholesterol fraction.

graphed on silica gel, and two substances were easily separated. The first, eluted with mixtures of petroleum ether and ether, was cholestanol from the infrared spectrum in carbon disulfide. The second material was eluted with ether-ethanol mixtures and had an infrared spectrum in potassium bromide identical with that of cholestane-3β,5α,6β-triol. Twice the weight of nonradioactive cholestanol was added to the cholestanetriol, and after a 3 transfer partition of the mixture between petroleum ether and 90 per cent methanol, cholestanetriol was obtained from the methanol phase. Crystallized from methanol, it melted at 238–242°; other investigators reported 237–239° (6).

In order to convert any epimeric Δ^4-cholestene-3-ols which might be present to $\Delta^{3,5}$-cholestadiene, another portion of the cholesterol from the chromatogram of the nonsaponifiable fraction was refluxed with 1 per cent hydrocholric acid in ethanol for 2 hours (7). Carrier $\Delta^{3,5}$-cholestadiene was added to the reaction product in a weight ratio of 10:1, and the mixture was chromatographed on alumina. The cholesterol so obtained was converted to cholestanetriol, and the latter was acetylated to yield cholestane-3β,5α,6β-triol 3,6-diacetate. Two recrystallizations from methanol afforded needles (m.p. 165–166°) which did not depress the melting point of authentic diacetate.

Cholestanol, Experiments II and III—Cholestanol is not separated from cholesterol in the chromatographic system used. It was isolated from the mother liquors remaining after the formation of cholesterol dibromide. These were diluted with ether and washed with sodium sulfite and were then refluxed with zinc in acetic acid. The product, a mixture of cholestanol and cholesterol, was acetylated and treated with perbenzoic acid. The mixture of acetates was chromatographed on alumina, and cholestanyl acetate was separated from epoxidized material. Carrier cholestanyl acetate was added (Experiment II, 139 c.p.m. per mg., after correcting for the carrier addition), and the mixture was saponified and chromatographed on alumina to yield cholestanol (154 c.p.m. per mg.).

TABLE III

Comparison of specific activities of Δ^5 and Δ^4-cholestenone before and after incubation with feces

Experiment IIIA				Experiment IIIB			
Δ^5-Cholestenone, 10,000 c.p.m./mg. (before incubation)				Δ^4-Cholestenone, 9700 c.p.m./mg. (before incubation)			
Chromatogram fraction	% unsaturated ketone	c.p.m./mg. (measured)	c.p.m./mg.* (calculated)	Chromatogram fraction	% unsaturated ketone	c.p.m./mg. (measured)	c.p.m./mg. (calculated)
8	55.0	5100	9100	9	71.5	6440	8900
9	31.6	2980	9000	10	35.7	3360	8900
10	13.4	1350	9200	11	26.5	2560	8850
11	11.5	1270	10000	12	8.0	1090	10000

* $[\text{c.p.m./mg.}]_{\text{calculated}} = \dfrac{[\text{c.p.m./mg.}]_{\text{measured}}\ (1 - x)\ [\text{c.p.m./mg.}]_{\text{coprostanol}}}{x}$,

where x = per cent of unsaturated ketone in the fractions and coprostanol is the contaminant.

TABLE IV

Purification of cholesterol-C^{14} (Experiment IV)

Procedure	Cholesterol c.p.m./mg.	Procedure	Cholesterol c.p.m./mg.
Fraction from chromatogram	190	Fraction from chromatogram	202
After "washout" with 300 mg. of coprostanol	218	After reflux with 1% HCl and "washout" with cholestadiene; diluted with carrier cholesterol	80
After "washout" with 200 mg. of cholestenone	244	Oxidation to cholestane-3β,5α,6β-triol	72*
Oxidation to cholestane-3β,5α,6β-triol	268*	Cholestane-3β,5α,6β-triol-3,6-diacetate	75
After "washout" of triol with cholestanol	262	Two additional crystallizations of the diacetate	78

* These values and those following have been corrected back to cholesterol.

More Polar Material—In all experiments these fractions were combined, and the radioactivity was measured.

Radioactivity Measurements[5]—Samples were dissolved in ethanol; portions containing 0.1 to 0.3 mg. of material were plated on 5 cm.^{2} nickel-plated stainless steel planchets and counted in a windowless gas flow counter. Measurements were made in triplicate and corrected to infinite thinness.

RESULTS AND DISCUSSION

In the conversion of cholestenone to cholesterol by feces, there was a distinct possibility that the radioactivity associated with cholesterol represented small amounts of highly radioactive steroid contaminants, since the reaction products are difficult to separate. However, purification of the cholesterol to constant specific activity together with "washout" procedures which would remove suspected contaminants showed that the conversion took place in measurable amounts. Although the quantity of cholesterol formed from cholestenone was small, it is probable that the conditions of the reaction could be modified to produce an increase in yield.

The principal transformation product of cholestenone was coprostanol (Table II), as reported by other investigators (8, 9, 10). The low conversions in Experiments IIIA and IIIB can probably be explained by the comparatively large quantities of cholestenone added, much of which was recovered unchanged. In Experiment III the isomeric cholestenones were compared as coprostanol precursors. The results were not as definitive as hoped for; however, there is an indication that Δ^4-cholestenone was converted in larger amounts. The possibility arises that cholesterol might be an intermediate in the conversion of cholestenone to coprostanol (10); however, under the experimental conditions of these studies, this did not appear to be the fact (Table II), since the specific activity of coprostanol is 3 to 6 times that of cholesterol. Cholestanol was a minor transformation product of cholestenone under the conditions that obtained (Table II). In contrast, Stokes *et al.* (11) and Harold *et al.* (12) found that cholestenone was largely transformed to cholestanol when the ketone was injected intravenously into rats; this conversion probably was accomplished in the liver.

A significant amount of cholestenone was transformed to coprostanone; in Experiment II, in which the saturated ketone was purified, coprostanone and coprostanol had the same specific activity.

Cholestenone itself was identified in the reaction products only in the experiments in which it was added to the medium. It has been found that 3 per cent of the unsaturated ketone can be detected by ultraviolet spectrometry when cholestenone is added to the appropriate eluates. Since no peak at 240 mμ was observed in the fractions which were subsequently shown to contain cholestenone (Experiments IA and II), this substance amounted to less than 3 per cent of these eluates.

A systematic examination of the cholestenone-containing fractions of Experiments IIIA and IIIB revealed that little Δ^4-cholestenone could have been present in the feces prior to the addition of the labeled ketone. Furthermore, this compound did not accumulate from other products during the incubation, since the specific activity of the cholestenone isolated was virtually unchanged from the original value (Table III). These results are in agreement with the conclusions of Cook *et al.* who found spectroscopic evidence of trace amounts of unsaturated ketone in human feces (13) and in rat feces only after ingestion of large amounts of cholesterol (14).

The above observation furnishes additional evidence that cholestenone is not a major intermediate in the conversion of cholesterol to coprostanol under the conditions of the experiments. This is in agreement with recent investigations in which cholesterol-3D,4-C^{14} was transformed to labeled coprostanol without loss of deuterium at C-3 (3, 15).

SUMMARY

1. The transformation of Δ^4-cholestene-3-one-4-C^{14} to cholesterol-C^{14} by suspensions of human feces has been demonstrated.

2. The principal conversion product of Δ^4-cholestene-3-one-4-C^{14} under the conditions of the experiment was coprostanol-C^{14} which was obtained in yields of 6, 36, 56, and 63 per cent. Other transformation products were coprostanone and cholestanol.

3. Cholestenone was not present in significant amounts in feces before or after the experiments.

Acknowledgment—The support and interest of Dr. T. F. Gallagher in this investigation are gratefully acknowledged.

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[5] These were carried out under the direction of our colleague, Dr. H. Leon Bradlow, to whom we are very grateful.

A Comparison of Methods for the Analysis of Estrone, Estradiol, and Estriol in Extracts of Human Urine*

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(Received for publication, April 29, 1958)

The purpose of this report is to describe experiments which test the accuracy and specificity of the procedures of Brown (1) and of Brown *et al.* (2) for the determination of estrone, estradiol-17β, and estriol in human urine. In two experiments, urine specimens were collected from oophorectomized-adrenalectomized women who had each received approximately 2 mg. of estradiol-17β-16-C^{14} intravenously. The urines were incubated with β-glucuronidase, extracted with ether, and the estrone, estradiol-17β, and estriol content of the ether extracts were estimated by the following methods: (*a*) reverse isotopic dilution; (*b*) determination of the radioactivity content of the peak tubes after countercurrent distribution; (*c*) the procedure of Brown (1), hereafter termed "Method A" (Experiment I), and of Brown *et al.* (2), hereafter termed "Method B" (Experiment II), which utilize the Kober color reaction; (*d*) determination of the radioactivity of the separated estrogen methyl ether fractions obtained before colorimetry. A third experiment was performed to test Method B at lower levels of estrogen excretion than in the first two experiments. A portion of the previously analyzed urine extract from the second experiment was diluted with an "inert" extract of urine obtained from an oophorectomized-adrenalectomized woman who had not received any estrogen. The amounts of estrogen added were measured by Method B and by the radioactivity of the separated methyl ether fractions. This was in effect a recovery experiment of the type commonly used to test the accuracy of an assay procedure, with the particular features that all of the various estradiol metabolites in normal proportion were added and the additional check of radioactivity measurement was possible.

In all of these experiments, the administration of radioactive estradiol, the collection of urine, the preparation of the preliminary extracts, and the radioactivity measurements were performed at the Sloan-Kettering Institute. The processing of the urine extracts by Methods A and B and colorimetric measurement were performed at the Clinical Endocrinology Research Unit, Edinburgh. The workers from the latter institution had no prior information concerning the estrogen content of the extracts which had been sent to them. The separated estrogen methyl ether fractions were returned to the Sloan-Kettering Institute for measurement of radioactivity.

* This investigation was supported in part by a grant from the American Cancer Society and a research grant (CY-3207) from the National Cancer Institute, National Institutes of Health, United States Public Health Service.

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EXPERIMENTAL

Preparation of Extracts

The urine after the addition of 1 mg. per ml. of Versene (disodium salt of ethylenediaminetetraacetic acid, Dow Chemical Company) was incubated with beef-liver glucuronidase[1] (300 units per ml.) at pH 5 and 37° for 5 days. At the end of incubation the urine at pH 5 was extracted continuously with ether for 48 hours. The ether extract was washed with sufficient saturated aqueous sodium bicarbonate solution so that the solution was alkaline. The ether layer (about 1 l.) was then washed six times, each time with 84 ml. of sodium bicarbonate solution. The combined aqueous phases were re-extracted with two portions of ether. Combined ether layers were washed twice, each with 25 ml. of water, the ether was removed, and the residue was dissolved in absolute ethanol. This solution was divided into portions for analysis by the various procedures.

Experiment I; Subject P (Female, Age 40)—This patient, who had metastatic cancer of the breast and had been adrenalectomized and oophorectomized, was removed from steroid therapy 48 hours before the study. No other steroid except the estrogen was given for the succeeding 2 days. The patient received 2.13 mg. of estradiol-17β-16-C^{14} (4,220,000 c.p.m.) intravenously in 5 per cent glucose solution containing sufficient ethanol to dissolve the hormone. The urine extract, prepared as above, was divided into four equal portions, each of which contained 273,000 c.p.m. To the portion used for analysis by reverse isotopic dilution the following pure carrier steroids were added: 30.4 mg. of estrone; 31.0 mg. of estradiol; 50.0 mg. of estriol. The carrier steroids were of the highest purity, as judged from their physical constants. The mixture containing extract and carrier was evaporated to dryness and distributed through 99 transfers in the system with 70 per cent methanol-30 per cent water as one phase and then carbon tetrachloride as the other phase. The contents of tubes Nos. 69 to 99 were combined and redistributed through 99 cycles in the system involving ethyl acetate-cyclohexane 1:1 as one phase and ethanol-water 1:1 as the other.

Estrone—The material contained in tubes Nos. 44 to 68 of the first countercurrent distribution was crystallized from absolute ethanol. Five successive recrystallizations yielded estrone; m.p. 256.5–259.5°. The specific activity was constant through

[1] Ketodase, obtained from the Warner-Chilcott Laboratories, a division of Warner-Lambert Pharmaceutical Company, New York, New York.

TABLE I

Comparison of methods for determination of estrone, estradiol-17β, and estriol in human urine

Subject and compound	R.I.D.*	Peak tube	Kober	Radioactivity of methyl ethers
	Amount per quarter of extract (μg.)			
Experiment I, Patient P				
Estrone	46	43	44	44
Estradiol	23	27	22	31
Estriol	37	36	27	39
Experiment II, Patient E				
Estrone	29	29	28	20
Estradiol	21	18	18	22
Estriol	70	61	61	69
	Amount added (μg.)		*Amount found (μg.)*	
Experiment III, Patient E + "inert" P†				
Estrone	2.6		2.4	1.3
Estradiol	1.8		1.4	1.7
Estriol	6.3		5.9	6.3

* Reverse isotopic dilution.

† Inert extract of urine, Patient P.

the final three crystallizations with an average value of 2840 c.p.m. per mg. The total radioactivity of estrone was, therefore, 2840 c.p.m. per mg. × 30.4 mg. = 86,400 c.p.m. The administered estradiol with a specific activity of 1,845,000 c.p.m. per mg. would be converted to estrone with a specific activity of 1,860,000 c.p.m. per mg. Therefore, the total weight of estrone present in the one-quarter portion of the extract was (86,400 c.p.m. × 1000)/(1,860,000 c.p.m. per mg.) = 46 μg. (Table I).

Estradiol—The material contained in tubes Nos. 62 to 75 from the second countercurrent distribution was combined and crystallized from ethanol. Four successive recrystallizations yielded estriol; m.p., 280–281°. The specific activity was constant through the final three crystallizations with an average of 1280 c.p.m. per mg. Estriol derived from the administered estradiol would yield a product with a specific activity of 1,742,000 c.p.m. per mg. Therefore, the total weight of estriol in the portion analyzed which corresponded to one-quarter of the extract was (64,000 c.p.m. × 1000)/(1,742,000 c.p.m. per mg.) = 37 μg. (Table I).

A portion of the estriol was converted to the 3-monobenzoate by treatment with benzoyl chloride in the presence of dilute sodium hydroxide at room temperature for 2.5 hours. The product was crystallized successively from ethyl acetate and from aqueous ethanol. The specific activity was constant through three recrystallizations with an average of 930 c.p.m. per mg. Estriol with a specific activity of 1280 c.p.m. per mg. should afford a monobenzoate with a specific activity of 940 c.p.m. per mg. The specific activity of this derivative was added assurance that the estriol was radiochemically pure.

Analysis for Estrogens from Radioactivity in Peak Tube of the Countercurrent Distribution

This was carried out as described by Beer and Gallagher (3) using the formula of Williamson and Craig (4). The results obtained for the one-quarter portion of the extract are shown in Table I.

Analysis by Method A—Another portion of the extract corresponding to one-quarter of the total was divided into two equal portions for duplicate determinations by Method A. Each portion was taken up in 400 ml. of ether and processed exactly as if it were the total ether extract of 200 ml. of hydrolyzed urine, as detailed in the description of the method (1). The separated fractions corresponding to the methyl ethers of estrone, estradiol, and estriol were each subdivided further into two equal portions. One of these was used for measurement by the Kober reaction; the other was evaporated to dryness by heating it in a water bath under a stream of nitrogen, after which it was returned for measurement of radioactivity. The mean values of the duplicate analyses by the Kober reaction were as follows: estrone, 9.3 μg.; estradiol, 4.6 μg.; and estriol, 5.8 μg. It had been established from recovery experiments that the average loss of estrogens in the extraction and fractionation process was approximately 15 per cent (5). Therefore, a correction factor of 100/85 was applied to these estimates. The results, which correspond to one-quarter of the total extract, are shown in Table I.

The estrone methyl ether fraction contained 69,500 c.p.m. calculated to the original one-quarter portion of the extract. From this value it was calculated that this fraction contained the equivalent of 37 μg. of estrone. The estradiol monomethyl ether fraction contained 27,400 c.p.m. calculated to the original one-quarter portion of the extract. From this value, the fraction contained the equivalent of 26 μg. of estradiol. The estriol monomethyl ether fraction contained 58,000 c.p.m. calculated to the original one-quarter portion of the extract. From this value the fraction contained the equivalent of 33 μg. of estriol. The values shown in Table I were obtained when these estimates from the radioactivity measurements were corrected for the expected losses in the extraction and fractionation procedures.

Experiment II; Subject E (Female, Age 65)—This patient also had metastatic cancer of the breast and had been adrenalectomized and oophorectomized. She received 2.15 mg. of estradiol-17β-16-C^{14} (1,890,000 c.p.m. per mg.) intravenously, in the same solution as that used in Experiment I. The urine extract, prepared as above, was divided into four equal portions, each of which contained 355,000 c.p.m. To the portion used for analysis by reverse isotopic dilution the following pure carrier steroids were added: estrone, 44.4 mg.; estradiol, 41.9 mg.; and estriol, 57.6 mg. A mixture containing extract and carrier was distributed in the same solvent systems used in the preceding experiment. The results of these distributions are shown in Figs. 1 and 2. Each of the separated compounds was purified to radiochemical homogeneity as before. The following results were obtained:

Estrone—This compound contained 1260 c.p.m. per mg., which corresponded to 29.3 μg. in the one-quarter portion of the extract examined.

Estradiol—This compound contained 930 c.p.m. per mg., which corresponded to 20.6 μg. in the one-quarter portion of the total extract.

Estriol—This compound contained 2160 c.p.m. per mg.,

which corresponded to 70.0 μg. in the one-quarter portion of the total extract.

These results are recorded in Table I.

Analysis by Method B—One-quarter of the extract was divided into two portions as before and each was processed separately by Method B. The purified extracts were analyzed in duplicate by the Kober reaction and also by their radioactivity content. The results corrected for expected methodological losses are shown in Table I.

Experiment III; Analysis at Low Levels of Estrogen Metabolites—A 24-hour urine collection was obtained from Subject P, 48 hours after she had been withdrawn from all steroid therapy (no steroid was administered during the collection). An extract was prepared from this "inert" urine, and a portion of it was analyzed by Method B with the following results, corrected for methodological losses: estrone, 0.17 μg.; estradiol, 1.47 μg.; and estriol, 0.73 μg. To another equal portion of this same urine extract was added a known portion of the urine extract from Subject E already described above. According to the previous analyses, the amount of each radioactive metabolite added was: estrone, 2.6 μg.; estradiol, 1.8 μg.; and estriol, 6.3 μg. The mixture ("inert" plus radioactive) was divided into two equal parts as before and processed in duplicate by Method B. Three-quarters of each of the separated estrogen methyl ether fractions, corresponding to three-eighths of the original mixture, was used for measurement by the Kober reaction. The remainder was used for radioactivity measurement. The results obtained by the Kober reaction, corrected for methodological losses, gave the total estrogen content of the mixture as estrone, 2.6 μg.; estradiol, 2.9 μg.; and estriol, 6.6 μg. The amounts of each radioactive metabolite added were calculated by subtracting the values for the inert component from these figures. The results are shown in Table I. Also shown are the figures calculated from the radioactivity measurements of the methyl ether fractions.

DISCUSSION

In general, the agreement between the results obtained by all the assay methods was highly satisfactory. Since the results obtained by reverse isotopic dilution appear unobjectionable on theoretical grounds, it follows that the other methods also give correct results under the conditions tested.

There were two instances in which the deviation of results seemed greater than could be accounted for by experimental errors. In Experiment I, the estriol value obtained by Method A was less than that obtained by the other methods. Subsequent to this experiment, it was shown that estriol fractions prepared from enzyme-hydrolyzed urine and processed by Method A contain substances which interfere in the Kober reaction. The lower estimate of estriol is consistent with an explanation on the basis of this interference. Method B is a modification of Method A which incorporates the saponification stage developed by Bauld (6). This additional purification step elimininates the substances present in extracts of enzyme-treated urine which interfere in the Kober reaction. It was for this reason that Method B was used in the subsequent experiments.

In Experiment III, the result obtained by measuring the radioactivity of the estrone methyl ether fraction was low. There is no immediately apparent explanation for this. The methyl ethers were evaporated to dryness for dispatch for radioactivity measurement. It is possible that an appreciable portion of the trace amount of estrone methyl ether might have been volatilized during this treatment.

The amounts measured by the Kober reaction in Experiments I and II were actually one-quarter, and those in Experiment III were three-eighths, of those shown in Table I. These figures give an indication of the sensitivity of the methods. However, caution is necessary in interpreting the results of Experiment III. They apply to estrogens added to urine but do not necessarily apply to similar levels of endogenous excretion. For example, there is still considerable doubt concerning the significance of the very low values obtained for the estrogen content of the "inert" extract used in the third experiment. Thus, although there is an indication that Method B is applicable at low estrogen levels, it would seem desirable to await further study before indiscriminate use of the method in this range.

It should be pointed out that the analyses reported here were performed on the extracts of urine which had already been hydrolyzed by β-glucuronidase. Methods A and B were originally developed for acid-hydrolyzed urine and all the data pertaining to their reliability were derived from this source (5). However, the purpose of these experiments was not to compare the efficiency of various hydrolytic procedures. It can be concluded from the results that, within the limits tested, Method B

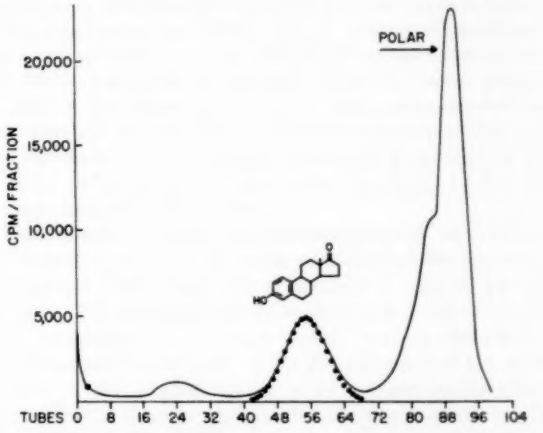

FIG. 1. Distribution of urine extract after an intravenous injection of estradiol-16-C^{14}. System: 70 per cent methanol-30 per cent water and carbon tetrachloride.

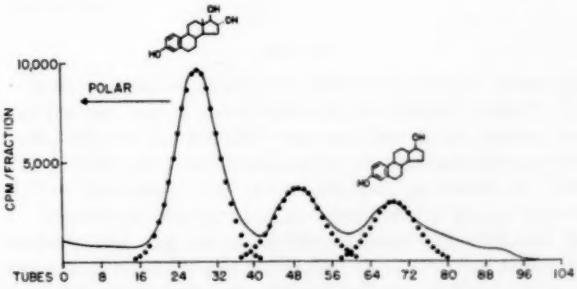

FIG. 2. Redistribution of polar steroids after an intravenous injection of estradiol-16-C^{14}. System: 50 per cent ethyl acetate-50 per cent cyclohexane and 50 per cent ethanol-50 per cent water.

is reliable for estimating estrone, estradiol-17β, and estriol in extracts of enzyme-hydrolyzed urine and in the presence of biochemically equivalent amounts of other metabolites of estradiol-17β. These findings add considerable weight to the other evidence on which the statements of reliability of the methods have been based (5).

SUMMARY

Urine extracts containing the metabolites of administered estradiol-17β-16-C^{14} were analyzed for estrone, estradiol, and estriol by the following methods: (*a*) reverse isotopic dilution; (*b*) determination of the radioactivity content of the peak tubes after countercurrent distribution; (*c*) the methods of Brown (1) or of Brown *et al.* (2) which utilize the Kober color reaction; (*d*) determination of the radioactivity content of the separated estrogen methyl ether fractions obtained by the method of Beer and Gallagher (3).

Satisfactory agreement between all these analyses was obtained.

Acknowledgments—The technical assistance of Miss Ruth Jandorek, Mrs. Rosemarie Lehman, and Mrs. Janet Blair is gratefully acknowledged.

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The Effect of Follicle-stimulating Hormone on the Biosynthesis *in Vitro* of Estradiol-17β from Acetate-1-C^{14} and Testosterone-4-C^{14}*

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(Received for publication, June 12, 1958)

The conversion of C^{14}-testosterone to C^{14}-labeled estrogens was shown by Heard (1) to take place in the pregnant mare. Baggett *et. al.* (2) and Wotiz *et al.* (3) have demonstrated this same conversion in human ovarian slices. The conversion of C^{14}-acetate to C^{14}-estradiol and estrone in dog ovary slices and homogenates has been described by Rabinowitz and Dowben (4).

The present study concerns the action of FSH,[1] administered *in vivo* or *in vitro*, on the converion of C^{14}-acetate and C^{14}-testosterone to C^{14}-estradiol-17β in dog ovary slices.

EXPERIMENTAL

Ovaries were obtained from two groups of mongrel female dogs. One group was designated anestrous. In this group the vulvae were atrophic, vaginal smears showed no cells, the uteri were small, thin, and pale, and the ovaries were small, compact, and whitish, and ranged in weight from 0.4 to 0.9 gm. The dogs of the second group were treated with 6 mg. of FSH[2] per day per dog for 11 days before they were killed. At death these animals had greatly swollen vulvae, vaginal smears showed abundant cornified cells, and the uteri were enlarged. The ovaries were large, had numerous follicles filled with yellowish fluid, and ranged in weight from 1.1 to 1.3 gm. Before the dogs were killed they were anesthetized with Nembutal, after which the ovaries were removed and placed immediately in a beaker of cracked ice. The capsules of the ovaries were then removed and with a Stadie slicer the ovaries were sliced to a thickness of approximately 0.5 mm. 400 to 500 mg. of tissue were transferred to Warburg vessels each of which contained 1 μc. (= 80 μg.) of testosterone-4-C^{14} (specific activity, 12.3 μc. per mg.),[3] 1 ml. of dog serum, and 1.5 ml. of bicarbonate-phosphate buffer at pH 7. The composition of the buffer was 0.065 M K_2HPO_4, 0.040 KH_2PO_4, 0.006 M $MgCl_2$, 0.006 M $KHCO_3$, 0.030 M nicotinamide, 0.0008 M diphosphopyridine nucleotide, 0.0029 M adenosine-5′-phosphate. In some of the incubations in which anestrous ovaries were used, there was, in addition, 1 mg. of FSH in the vessels. The atmosphere was 5 per cent CO_2 in oxygen, and the mixture was incubated in a 37.5° water bath for 3 or 4 hours with shaking. At the end of the incubation 1 mg. of estradiol-17β was added as carrier to each vessel.

The liquid phase was diluted with 10 volumes of physiological saline and extracted four times with 50 ml. of methylene chloride. The tissue was dried in acetone, frozen in liquid nitrogen, pounded to fine flakes in a metal mortar, and extracted with the methylene chloride used to extract the corresponding liquid. The remainder of the extraction procedure is outlined in Diagram 1. After extraction, the tissue was dried further in a vacuum desiccator and weighed.

The phenolic fraction was chromatographed on Schleicher and Schuell paper in a formamide-*o*-dichlorobenzene system (5). The estradiol strip was located and eluted with 95 per cent ethanol. The chemical estimation of estradiol was determined by a modification of the colorimetric reaction described by Lieberman and Tagnon (6). After determination of the specific activity of the isolated carrier, a 24 transfer countercurrent separation was done on the estradiol fraction with the use of a 50 per cent methanol-CCl_4 system. After removal of aliquots for analysis, additional estradiol was put in and the mixture was recrystallized from ethyl acetate-petroleum ether. The specific activity of both the crystals and the mother liquor was ascertained.

RESULTS

In the samples stimulated by FSH, the formation of estradiol was 0.6 per cent of the C^{14}-testosterone initially added. The radiopurity of the estradiol was indicated by a constant specific activity obtained through paper chromatography, countercurrent separation, and recrystallization, as shown in Table I. In addition, the estradiol, as determined by the colorimetric method, and the corresponding radioactivity, followed the theoretical curve for the 24 transfer countercurrent separation in all the experiments, one of which is shown in Fig. 1.

The transformation of testosterone-C^{14} to estradiol by ovarian slices is accelerated by FSH whether this is administered by injection into the animal or is added *in vitro*. Table II is a

* This work was supported by a grant from the Damon Runyon Memorial Fund for Cancer Research, Inc.

† United States Public Health Postgraduate Fellow.

[1] The abbreviation used is: FSH, follicle stimulating hormone.

[2] We are grateful to Dr. Steelman and Mr. Bunding of Armour and Company for making available generous quantities of FSH.

[3] Testosterone-4-C^{14} was obtained from Tracerlab, Inc. It was added to each Warburg vessel as an alcohol solution before the experiment and the alcohol was then evaporated.

DIAGRAM 1

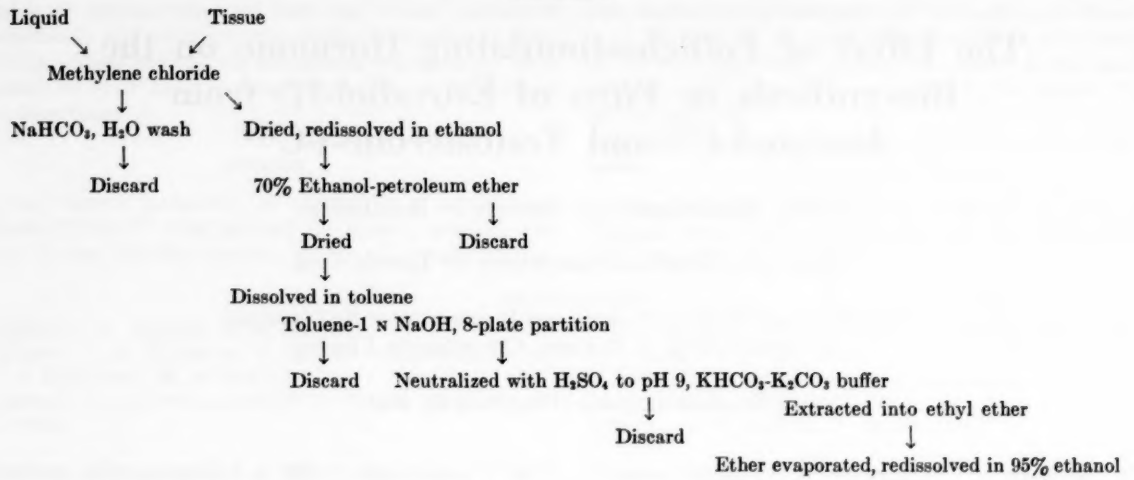

TABLE I

Specific activity of estradiol

Experiments 1 and 2 both involved the use of ovaries of dogs treated with FSH. Additional estradiol was put in for countercurrent distribution and again for recrystallization, but the specific activities are corrected for comparison.

Method	System	Experiment 1	Experiment 2
		c.p.m./mg. estradiol	*c.p.m./mg. estradiol*
Paper chromatography	Foramide-*o*-dichlorobenzene	5000	4800
24 transfer countercurrent	50% methanol-CCl_4	4800	4600
Recrystallization	Ethyl acetate-petroleum ether	4800	5100

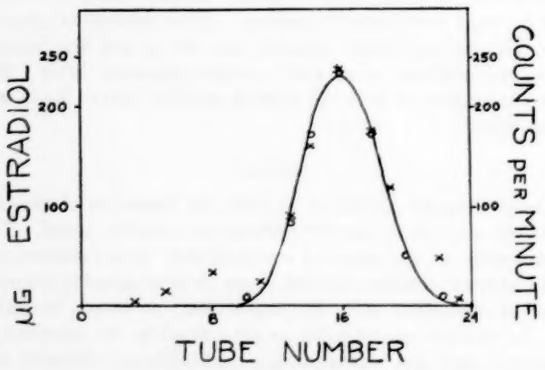

FIG. 1. The theoretical curve based on the calculations of Craig (7), is drawn in. The theoretical and the two experimental maxima were made to coincide. O, colorimetric estimation of estrogen; X, radioactivity.

TABLE II

Comparison of activities

Activities are expressed as counts per minute per mg. of estradiol per mg. of ovarian tissues, dry weight. The differences between Group I and Groups II, III, IV, and V are significant; $p < 0.02$ for Group II and $p < 0.01$ for the others.

Group I Anestrous	Group II Anestrous and FSH *in vitro*		Group III FSH *in vivo*	Group IV FSH *in vivo* and *in vitro*	Group V Anestrous and HCG* *in vitro*
c.p.m.	*c.p.m.*	*mg. FSH*	*c.p.m.*	*c.p.m.*	*c.p.m.*
11.8	51.5	10.0	95.4	74.2	25.8
21.8	85.5	0.5	63.2	68.9	41.6
10.9	24.6	1.0	50.0	118	70.8
11.5	52.8	1.0	73.8		59.0
9.8	42.5	1.0			12.8
14.4	13.5	1.0			55.5
23.1	26.0	1.0			
16.8					
Mean ± s.e.					
15.0 ± 1.8	42.4 ± 9.1		70.9 ± 9.4	90.7 ± 7.2	44.3 ± 8.9

* HCG, human chorionic gonadotropin.

summary of the effect of FSH on this conversion. The results are given in terms of dry weight of ovarian tissue. The effect of the FSH added *in vitro* seems to be maximal in these studies since no difference in conversion was noted between duplicate vessels containing 0.5 mg. and 10 mg. of FSH. FSH added *in vitro* to ovary slices from FSH-treated dogs produced no additional stimulation and no inhibition.

When chorionic gonadotropin instead of FSH was added *in vitro* to anestrous ovary slices, there was a similar stimulation of activity.

Carboxyl-labeled Acetate C^{14} as Precursor[4]—Carboxyl-labeled

[4] Acetate-1-C^{14}, specific activity (*a*) 1 mc. per mmole and (*b*) 5 mc. per mmole, was obtained from Nuclear Chicago Corporation.

acetate was studied in the incubation system described above. The extraction and the isolation of the estradiol was identical. No activity could be found in the estradiol in the experiments in which acetate was used as the precursor. A total of 42 experiments was performed with both FSH and chorionic gonadotropin administered to the dogs or added to the incubation vessel. Some experiments were performed with acetate that had a specific activity of 5 mc. per mmole when acetate of 1 mc. per mmole afforded no evidence of conversion. Increasing the volume of acetate in the incubation vessel to 65 μc. of acetate with a specific activity of 5 mc. per mmole did not affect the result. In two negative experiments, the cholesterol isolated as the digitonide was found to be radioactive, with a specific activity that indicated the conversion of approximately 0.1 per cent of the acetate to cholesterol.

DISCUSSION

The failure of the data to show incorporation of C^{14}-acetate into estradiol-17β under any of our conditions does not imply that this pathway does not exist in the ovary. On the contrary, the evidence that a C^{19} compound is a precursor of estradiol reinforces the role of acetate as an eventual carbon source. Acetate, however, enters into a multiplicity of reactions of which steroidogenesis is but a minor path. It is possible that experiments performed with an even higher specific activity of acetate-C^{14}, or with an inhibitor to the alternate pathways of acetate utilization, would show the incorporation of acetate into estradiol. The repeated isolation of nonradioactive estradiol from acetate incubation mixtures of high radioactivity afforded evidence that the estradiol purification was adequate. The isolation of radioactive cholesterol from such mixtures indicated that the tissue was metabolically active.

By contrast, in all experiments there was evidence of the conversion of testosterone-4-C^{14} to estradiol-17β. The radiopurity of the product was indicated by the constant specific activity of estradiol through paper chromatography, countercurrent separation, and recrystallization. The administration of FSH to dogs increased the extent of conversion of testosterone to estradiol. This experiment showed that a rate-limiting reaction involved in this conversion is stimulated by FSH. The response of anestrous ovaries to FSH *in vitro* was evidence that this reaction is prompt and is not the result of increased enzyme synthesis due to growth. The fact that stimulation *in vitro* of ovaries from FSH-treated animals did not lead to further incorporation suggests that the tissue was already stimulated to the maximal degree.

FSH and human chorionic gonadotropin have long been known to increase estrogen production. The implication of gonadotropin in the aromatization of testosterone suggests that this reaction is of physiological importance in estrogen biosynthesis.

SUMMARY

1. The conversion *in vitro* of testosterone-4-C^{14} to C^{14}-estradiol-17β in dog ovary slices is stimulated by follicle-stimulating hormone administered *in vivo* or *in vitro*.

2. The conversion of acetate-1-C^{14} to C^{14}-estradiol-17β could not be demonstrated.

3. The role of follicle-stimulating hormone is estrogen biosynthesis is discussed.

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Mevalonic Kinase: Purification and Properties*

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(Received for publication, June 18, 1958)

In 1956 Tavormina *et al.* reported that mevalonic acid, which had just been isolated as a growth factor for a Lactobacillus and which bears a close structural relationship to β-hydroxy-β-methylglutaric acid, is an extremely efficient precursor of cholesterol (1). Since then, reports from several laboratories have established that (*a*) only one of the isomers of mevalonic acid is biologically active (1); (*b*) the carboxyl group of mevalonic acid is not incorporated into cholesterol or squalene (2); (*c*) carbon 2 of mevalonic acid, the carbon next to the carboxyl group, is not incorporated into the angular methyl groups of squalene, but is located entirely in the main chain of squalene (3–5); (*d*) the over-all conversion of mevalonic acid to squalene requires ATP, Mn^{++} and a reduced pyridine nucleotide as cofactors (6, 7); and (*e*) the "biological isoprene unit," *i.e.* the derivative of mevalonic acid which undergoes condensation, is not derived from this acid by oxidative reactions (8). When a crude extract of yeast was fractionated (9) two fractions were obtained, both of which are required for the conversion of mevalonic acid to squalene. Fraction A, when incubated with mevalonic acid, ATP and Mn^{++}, forms an unidentified compound which can be subsequently converted to squalene by Fraction B in the presence of a reduced pyridine nucleotide. In a previous communication, it has been reported that Fraction A catalyzes the phosphorylation of mevalonic acid by ATP (10). This enzyme has now been partially purified and has been demonstrated to be a kinase which catalyzes the formation of phosphomevalonic acid as follows: mevalonic acid + ATP → phosphomevalonic acid + ADP. The purification procedure, the identification of the reaction products, and some of the properties of the enzyme are reported in this paper.

EXPERIMENTAL

The samples of mevalonic acid used were the gift to Dr. K. Bloch's laboratory from Dr. J. M. Sprague of the Merck Sharp and Dohme Laboratories, and the author is deeply indebted to them for their generosity. ATP labeled in the 2 terminal phosphorus atoms was prepared by Dr. A. Phillips by oxidative phosphorylation with rat liver mitochondria and AMP. The phosphoenolpyruvic acid and pyruvic kinase were the gift of Dr. E. Keller and were originally purchased from the Boellinger Company, Germany. The various nucleotides were crystalline samples of the Pabst Laboratories. Lactic dehydrogenase was purchased from the Nutritional Biochemicals Corporation.

Phosphomevalonic acid was separated from mevalonic acid by paper chromatography. The reaction mixture, usually 0.1 ml., was inactivated by heating for 2 minutes in a boiling water bath, transferred quantitatively onto a strip of Whatman No. 1 filter paper (1.5 inches wide), and chromatographed with *n*-butanol-HCOOH-H_2O (77:10:13 by volume). The nucleotides, inorganic phosphate, phosphomevalonic acid and mevalonic acid have R_F values of 0.0, 0.15, 0.15, and 0.75 respectively. The inorganic phosphate and phosphomevalonic acid can be separated by rechromatographing with methanol-ammonia-water (60:10:30 by volume). In this system, phosphomevalonic acid has an R_F value of 0.75, whereas the inorganic phosphate remains at the origin. For quick assay of enzyme activity during purification, the chromatograms were counted in a strip counter (Nuclear-Chicago Corporation). For more accurate purposes, the peaks were eluted and counted in a windowless flow counter. The ratio of P^{32}/C^{14} in the samples was determined by counting the samples in a thin window counter both with and without an aluminum window. The efficiency of counting for the 2 isotopes under these conditions was determined from standard samples.

RESULTS

Purification of Mevalonic Kinase—13 l. of yeast autolysate (9) were centrifuged for 30 minutes at 30,000 × *g* and the supernatant fluid was brought to 45 per cent saturation with ammonium sulfate by the addition of solid ammonium sulfate. The precipitate was collected by centrifugation (50 minutes at 23,300 × *g*) and was found to contain all the mevalonic kinase activity. All subsequent operations were carried out at 4°C and only deionized water was used. The precipitate was suspended with 900 ml. of phosphate buffer (0.03 M, pH 7.2) and centrifuged in the Spinco preparative ultracentrifuge (30 minutes at 104,000 × *g*). Both the precipitate and the fatty layer were discarded. The clear supernatant fluid (900 ml.) was diluted to 3 l.; 500 ml. of a 1 per cent solution of protamine sulfate (at room temperature) were added slowly and the precipitate was removed by centrifugation. The solution was again fractionated with solid ammonium sulfate. Most of the activity was found in fractions between 35 and 55 per cent saturation with ammonium sulfate. These fractions were combined and dialyzed against water overnight. 1 N acetic acid was added to an aliquot to lower the pH to 5 and the precipitate was removed by centrifugation. The solution was dialyzed overnight against 30 volumes of phosphate buffer (0.01 M, pH 6.8) and then against water. A considerable amount of precipitate was formed. This was removed by centrifugation (10 minutes at 23,300 × *g*). 115 ml. of the supernatant fluid (pH 6.2, 8.1 mg. of protein per ml.) were stirred gently for 10

* This work was supported by a grant-in-aid from the National Science Foundation and was carried out during the tenure of a Scholarship for Cancer Research from the American Cancer Society.

minutes with 50 ml. of calcium phosphate gel (11) (29 mg. per ml.). The gel was removed by centrifugation and the supernatant fluid was treated once more with gel. The gel was combined and eluted first with 40 ml. of 0.05 M phosphate buffer (pH 7.2) and then with 40 ml. of 0.2 M phosphate buffer (pH 7.4). To the combined eluates was added solid ammonium sulfate to 80 per cent saturation. The precipitate was collected by centrifugation, redissolved in 9 ml. of phosphate buffer (0.1 M, pH 6.8) and dialyzed overnight against 4 l. of phosphate buffer (0.001 M, pH 6.8). An aliquot of this dialyzed solution (11 ml. out of 12.5 ml.) was fractionated with a saturated solution of ammonium sulfate (saturated at 0°) and several fractions were collected. These were dialyzed overnight against 100 volumes of tris(hydroxymethyl)aminomethane (0.01 M, pH 7.4), and stored at −10°. The purification thus achieved is summarized in Table I.

Some Properties of Mevalonic Kinase—The most highly purified preparation was free of ATPase or myokinase activity and was used for all the results reported hereafter.

The enzyme is stable for several months at −10°. It is not activated by the addition of sulfhydryl compounds. However, it is inhibited by *p*-chloromercuribenzoate (83 per cent inhibition at 10^{-4} M) and this inhibition is completely reversed by the addition of reduced glutathione.

A divalent metal ion is required for activity. At low concentrations, Mn^{++} is by far the most active, and Mg^{++} is less active than either Zn^{++} or Co^{++}. However, Mg^{++} becomes increasingly active at higher concentration and, at high enough concentration, becomes the most active metal ion for this reaction. These results are shown in Fig. 1.

The enzyme is active over a wide range of pH, with maximal activity at pH 6.4 to 6.7. At the two extremes tested (pH 5 and 9), the enzyme still retains more than half of the maximal activity.

The requirement for ATP is shown in Fig. 2. As is the case with many kinases, mevalonic kinase is saturated only at high ATP concentration. ATP cannot be replaced by ADP. It can, however, be substituted for by GTP, CTP, or UTP. These crystalline triphosphates were tested at two different concentrations (20 μmoles per ml. and 4 μmoles per ml.) and were found to be of approximately the same activity. Further experiments, however, showed that although the enzyme appears to function equally well with any of the four nucleotides, it utilizes ATP preferentially in the presence of a mixture of ATP and UTP. In these experiments, the enzyme was incubated with 2-C^{14}-mevalonic acid, Mn^{++}, and mixtures of ATP^{32} and UTP. The ratio of ATP^{32} to UTP varied from one reaction mixture to another, but the total amount of nucleotides was kept constant. Phosphomevalonic acid was isolated, and the amounts of P^{32} and C^{14} in the samples were determined. The results are shown in Fig. 3. The C^{14} data gives the total amounts of phosphomevalonic acid formed and the P^{32} data give the amounts of phosphomevalonic acid that were formed from ATP. It can be seen that ATP is used preferentially over UTP. Furthermore, in this series of experiments, the enzyme becomes saturated with ATP at 10^{-2} M ATP, whereas in the absence of UTP, it is saturated only at 2×10^{-3} M ATP (Fig. 2). Thus, UTP, aside from being capable of substituting for ATP, also exerts a stimulatory effect on the utilization of ATP by the enzyme. The mechanism of this stimulation is not clear.

If DL-mevalonic acid is incubated with an excess of the enzyme and ATP and Mn^{++}, only half of the added mevalonic acid is phosphorylated. The residual acid cannot be converted to squalene when incubated with dialyzed yeast extract in the presence of ATP, Mn^{++}, and DPNH (9), whereas the phosphomevalonic acid can be converted to an extent of 60 to 70 per cent. It is thus concluded that mevalonic kinase is specific for the isomer of mevalonic acid which can serve as precursor of squalene and cholesterol.

Identification of Reaction Products—The identity of phosphomevalonic acid was established from the following information. It is formed from both carboxyl-labeled and C_2-labeled mevalonic acid with a yield of approximately 50 per cent from racemic mixtures of mevalonic acid. The carboxyl group of mevalonic acid must therefore be retained. When prepared with ATP labeled in the 2 terminal phosphorus atoms, the compound contains P^{32} and mevalonic acid in a 1:1 ratio as shown in Fig. 3. The compound must therefore be either a phosphate of mevalonic acid or an ADP derivative of this acid. The latter possibility is ruled out by the fact that the compound does not contain a chromophore that absorbs in the ultraviolet. This phosphate of mevalonic acid is quite stable to both acid and base. Heating for 30 minutes in a boiling water bath with 1 N KOH causes no change in its chromatographic behavior or in the efficiency of its further conversion to squalene by rat liver homogenate. Heating with 1 N HCl under identical conditions causes some changes in these properties without hydrolysis. This is shown by the fact that when the acid-treated material is rechromatographed, neither inorganic phosphate nor free mevalonic acid can be detected. That some change has taken place is indicated by the appearance of a broad and diffuse band in the chromatograms of this acid-treated material, whereas the phosphomevalonic acid only gives a narrow band under identical conditions. The efficiency of its conversion to cho-

TABLE I

Summary of purification procedure

Stage of purification	Specific activity	Total activity*
	units/mg.†	units†
Crude extract	10	2.2×10^6
Spinco supernatant	30	2.0×10^6
Protamine sulfate supernatant	60‡	3.6×10^6‡
Second $(NH_4)_2SO_4$; 35–55% saturation	90	3.5×10^6
pH 5 treatment	100	1.1×10^6
Third $(NH_4)_2SO_4$ fractionation:		
40–45% saturation	440	0.38×10^6
50–55% saturation	700	0.26×10^6
55–60% saturation	480	0.15×10^6

* Calculated for the total volume of crude extract.

† 1 unit is defined as 1 μg. of mevalonic acid phosphorylated per hour at 30° and under optimal conditions of pH, ATP, and Mn^{++} concentrations.

‡ The increase in total activity may be caused by the removal of nucleic acids which may bind Mn^{++} and thus inhibit the enzyme. It is also possible that the recorded activity of the first two fractions was inaccurate. These fractions contain considerable activity for the further transformation of phosphomevalonic acid and were assayed by the disappearance of mevalonic acid rather than by the accumulation of phosphomevalonic acid.

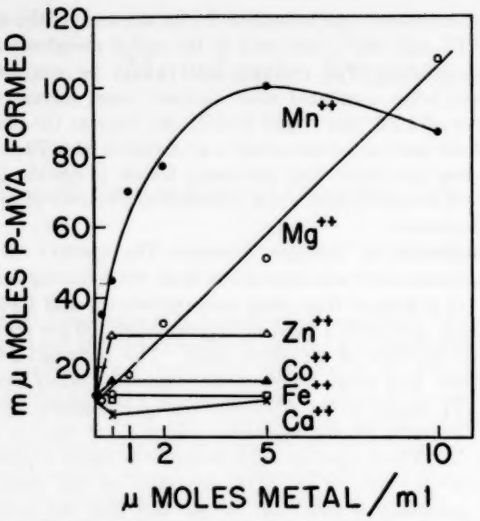

FIG. 1. Metal requirement for formation of phosphomevalonic acid (P-MVA). Each reaction mixture contained in a total volume of 0.1 ml.: 5 μl. of enzyme, 2 μmoles of ATP, 10 μmoles of phosphate buffer (pH 6.7), 1 μmole of 2-C^{14}-mevalonic acid and metal ion as shown in graph. Incubation was carried out at 30° for 1 hour.

lesterol by rat liver homogenate is also diminished by approximately 60 per cent. The stability of this compound and its failure to react with hydroxylamine eliminates the possibility that it is a carboxylphosphate, thus limiting the position of the phosphate group to one of the two hydroxyl groups in mevalonic acid.

Since one of the products of this enzymatic reaction is phosphomevalonic acid, it is expected that ADP would also be formed. This is confirmed by coupling the reaction to pyruvic kinase and lactic dehydrogenase. In such a system, the formation of

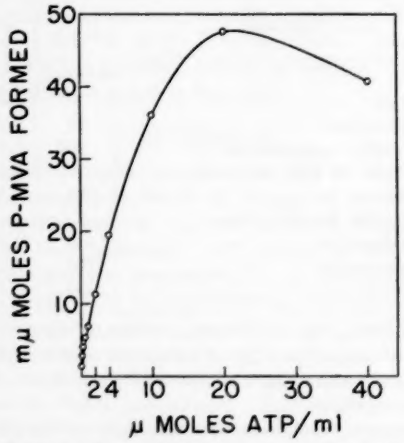

FIG. 2. ATP requirement for formation of phosphomevalonic acid (P-MVA). Each reaction mixture contained in a total volume of 0.1 ml.: 1 μmole of 2-C^{14}-mevalonic acid, 0.5 μmole of Mn^{++}, 10 μmoles of phosphate buffer (pH 6.7), 5 μl. of enzyme and ATP as indicated in graph. Incubation was carried out at 30° for 40 minutes.

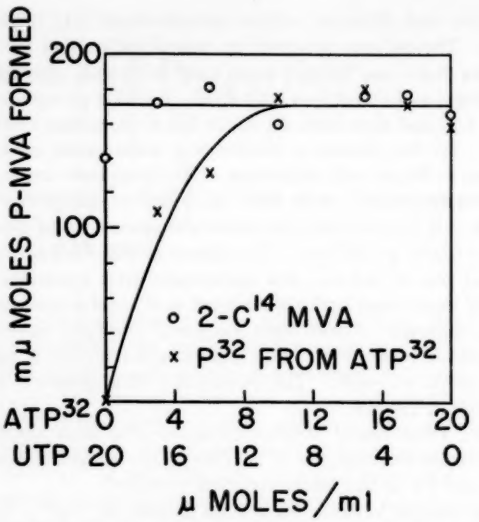

FIG. 3. Preferential utilization of ATP in the presence of UTP in formation of phosphomevalonic acid (P-MVA). Each reaction mixture contained in a total volume of 0.1 ml.: 1 μmole of 2-C^{14}-mevalonic acid, 0.5 μmoles of Mn^{++}, 10 μl. of enzyme, 10 μmoles of phosphate buffer (pH 6.7) and nucleotides as shown in graph. The measurements of specific activity (as measured in the windowless flow counter) of the mevalonic acid and ATP^{32} used were 5310 c.p.m. per μmole and 9432 c.p.m. per μmole, respectively. Incubation was carried out at 30° for 30 minutes.

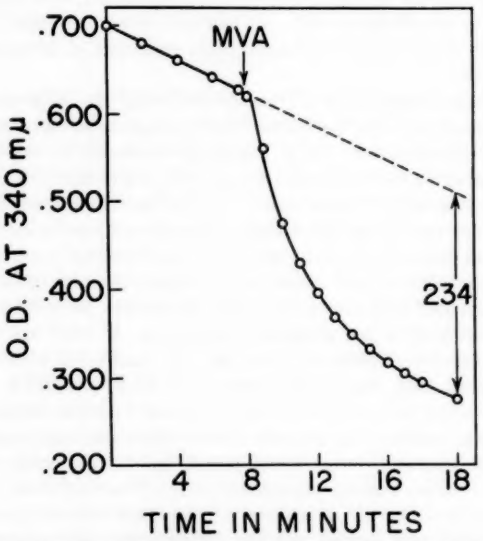

FIG. 4. Spectrophotometric determination of ADP formation after addition of mevalonic acid (MVA). The reaction mixture at the beginning of the experiment contained in a volume of 3.4 ml.: 10 μmoles of phosphoenolpyruvic acid, 20 μmoles of ATP, 100 μmoles of Mg^{++}, 340 μmoles of phosphate buffer (pH 6.8), 0.39 μmoles of DPNH, 0.1 ml. of mevalonic kinase, 30 μg. of crystalline pyruvic kinase and a large excess of lactic dehydrogenase. At 7 minutes and 45 seconds, 0.1 ml. of a neutral solution containing 270 mμmoles of DL-mevalonic acid was added.

ADP would be accompanied by the formation of pyruvate from phosphoenolpyruvic acid and consequently the oxidation of DPNH. This latter change can be followed conveniently in a Beckman spectrophotometer. The result of such an experiment is shown in Fig. 4. The amount of racemic mevalonic acid added was 270 mμmoles and the amount of DPNH oxidized, after correction for the blank oxidation, was 132 mμmoles, thus indicating that for each mole of the active isomer of mevalonic acid added, there is the formation of 1 mole of ADP.

DISCUSSION

Mevalonic kinase shows very similar properties to other kinases. It is saturated only at relatively high concentration of ATP and it requires a divalent metal ion for activity. The concentration at which these ions exert their optimal effect is worth noting. Thus, at 5×10^{-4} M, Mn^{++} already exerts half of the full activity, Zn^{++} and Co^{++} show some activity, whereas Mg^{++} is almost totally inactive. As the concentration increases, however, Mg^{++} becomes the most active metal ion for this reaction. Another interesting property of this enzyme is its lack of specificity with regard to the nucleotide. To the best of this author's knowledge, there is only one kinase, phosphofructokinase, which has been shown to be lacking in specificity for the nucleotides (12).

As mentioned earlier, the phosphomevalonic acid formed in this reaction can be further converted to squalene, but only in the presence of ATP. Recently, Phillips *et al.* have reported that, in the presence of ATP, phosphomevalonic acid is converted to another derivative of mevalonic acid which can subsequently give rise to squalene in the absence of ATP (13). It is thus concluded that phosphomevalonic acid is the first intermediate in the transformation of mevalonic acid to squalene.

One may ask what is the purpose of this phosphorylation of mevalonic acid by ATP. In a previous communication from this laboratory (8), it has been reported that the "biological isoprene unit" which undergoes condensation possesses terminal methylene groups which arise by the elimination of water or phosphate from mevalonic acid or phosphorylated derivatives of this acid. Thus one may put forth a chemical argument as a rationalization of the phosphorylation of mevalonic acid, namely, that phosphate esters are better leaving groups than hydroxyl groups and that the phosphorylation of mevalonic acid facilitates the formation of the terminal methylene groups in the "biological isoprene unit." Similar reactions where the elimination of a hydroxyl group is accomplished by phosphorylation followed by dephosphorylation have been postulated in the transformation of homoserine to threonine (14) and of DPNH-X to DPNH (15).

SUMMARY

Mevalonic kinase has been partially purified from an extract of yeast. It catalyzes the formation of adenosine diphosphate and phosphomevalonic acid from adenosine triphosphate and the biologically active isomer of mevalonic acid. Adenosine triphosphate can be replaced by the triphosphate of guanosine, cytidine or uridine, but not by adenosine diphosphate. The enzyme also requires a divalent metal ion for activity and is inhibited by *p*-chloromercuribenzoate. The role of this enzyme has been discussed.

Acknowledgment—The author wishes to thank Dr. K. Bloch and his coworkers for making their results available before publication as well as for many helpful discussions.

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Purification and Properties of Tyrosine-activating Enzyme of Hog Pancreas*

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(Received for publication, May 12, 1958)

A reaction between ATP and amino acids, which may be the first stage in protein synthesis, has been called amino acid activation by Hoagland (1). The proposed mechanism (2) of this reaction is shown in Equation 1.

$$\text{ATP} + \text{amino acid} + \text{enzyme} \rightleftharpoons [\text{enzyme-amino acyl-AMP}] + \text{P-P}^1 \quad (1)$$

Activation reactions of this type are catalyzed by enzymes from various sources (2–6) and specific activating enzymes may be required for each amino acid (2). The first highly purified, activating enzyme was a tryptophan-activating enzyme from beef pancreas, studied by Davie *et al.* (7). The isolation and properties of a tyrosine-activating enzyme from hog pancreas (8) are described in this report.

EXPERIMENTAL

The hydroxamic acid assay was similar to that described previously (5). The reaction mixture contained 5 μmoles of L-tyrosine; 30 μmoles of dipotassium ATP (Pabst Laboratories), adjusted to pH 7.5 with potassium hydroxide; 30 μmoles of magnesium chloride; 100 μmoles of Tris buffer, pH 7.5; 3000 μmoles of hydroxylamine hydrochloride adjusted to pH 7.0 with potassium hydroxide just before use; approximately 60 μg. of purified enzyme; and water to a final volume of 3.0 ml. The mixture was shaken in a Dubnoff metabolic shaker for 1 hour at 37°.

The reaction mixture for the P-P exchange assay contained 1 μmole of tyrosine; 10 μmoles of dipotassium ATP (crystalline, Pabst Laboratories), adjusted to pH 7.5 with potassium hydroxide; 10 μmoles of magnesium chloride; 100 μmoles of glycine buffer, adjusted to pH 8.5 with potassium hydroxide; 5 μmoles of radioactive potassium P-P (containing approximately 200,000 c.p.m.); approximately 30 μg. of purified enzyme; and water to make a final volume of 1.0 ml. The mixture was incubated at 37° for 10 minutes. The radioactivity in ATP was determined as before (5). The percentage of exchange and conversion to μmoles were calculated as described by Davie *et al.* (7).

A unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmole of hydroxamide per hour. Specific activity is given in units per mg. of protein. Protein was determined by light absorption (9). These determinations agreed with those obtained by the method of Lowry *et al.* (10).

Calcium phosphate gel (11) was aged several months before use and contained 30 mg. of dry material per ml. Salt-free hydroxylamine was prepared by the method of Hurd and Brownstein (12). This method utilizes the low solubility of sodium chloride in butanol for preparing the salt-free product. Tris-ATP and Tris-P-P were prepared by passing a solution of the respective sodium salt slowly through a column of Dowex 50 resin in the hydrogen form and adjusting the eluate to the desired pH with Tris buffer (free base).

Spectrographically pure rubidium chloride was purchased from the Jarrell-Ash Company, Newtonville, Massachusetts; DL-isomers of 3,4-dihydroxyphenylalanine, 3-aminotyrosine, *o*-tyrosine, *m*-tyrosine, *N*-chloroacetyltyrosine, and 3,5-diiodotyrosine were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio; L-*p*-fluorophenylalanine and tyramine from California Foundation for Biochemical Research, Los Angeles, California; DL-tyrosine amide and D-tyrosine from Mann Research Laboratories, New York, New York; and 3-nitrotyrosine from Delta Chemical Works, New York, New York. DL-2,5-Dihydroxyphenylalanine was a gift from Dr. A. Neuberger, National Institute for Medical Research, London, England.

Enzyme Preparation—Hog pancreas was defatted by hand in the cold room and 150-gm. portions were homogenized for 2 minutes with 550 ml. of acetone at −15° in a Waring Blendor. The mixture was filtered on a Buchner funnel. The cake was homogenized a second time in 550 ml. of acetone and filtered again. Two such filter cakes were combined and homogenized for 30 seconds with 550 ml. of peroxide-free ether at −15°. The final filter cake was dried at room temperature as rapidly as possible and the fine powder stored at −20°.

Fraction I—30 gm.[2] of acetone powder were ground in an ice-cold mortar with the addition of small portions of 0.02 M Tris buffer, pH 7.5.[3] The temperature was kept at 4° at all times during the fractionation, except where otherwise noted. The wet slurry was suspended in a total volume of 300 ml. of the same buffer and stirred occasionally for 20 minutes. The mixture was centrifuged at 70,000 × *g* for 60 minutes (Spinco preparative centrifuge, rotor No. 30), and the supernatant was decanted through glass wool.

* This work was supported in part by a grant from the National Science Foundation.

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[1] The abbreviations used are: P-P, pyrophosphate; Tris, tris-(hydroxymethyl)aminomethane.

[2] The amount of enzyme activity varies somewhat with the acetone powder used. Therefore, in some cases, as much as 40 gm. of acetone powder were treated as described to obtain a Fraction I containing 4 units per ml.

[3] The Tris buffer was adjusted to pH 7.5 at 20°.

Fraction II—To the clear supernatant (220 ml.) was added 40 ml. of calcium phosphate gel. The gel had previously been centrifuged and the supernatant discarded to minimize dilution of the enzyme extract. The slurry was adjusted to pH 7.5 and stirred gently for 30 minutes. It was then centrifuged at 3500 × *g* for 15 minutes. The supernatant solution was discarded. The gel was suspended in 110 ml. of 0.2 M potassium phosphate buffer, pH 6.0, and stirred gently for 30 minutes. The slurry was centrifuged as before and the supernatant discarded. The gel was then mixed with 110 ml. of 2.0 M potassium phosphate buffer, pH 8.1, and homogenized in a Potter-Elvehjem homogenizer if any lumps were apparent. The mixture was stirred for 30 minutes and then centrifuged for 60 minutes at 70,000 × *g*. The gel was discarded and the supernatant dialyzed with stirring for 18 hours against two changes of 2 l. of 0.02 M Tris buffer, pH 7.5.

Fraction III—To the dialyzed supernatant (200 ml.) were added 120 gm. of solid ammonium sulfate. The pH was maintained at 8.0 (measured on a 1:5 dilution) by the addition of cold N potassium hydroxide. After standing for 2 to 3 hours, the mixture was centrifuged and the supernatant discarded. The precipitate was dissolved in 10 ml. of 0.1 M Tris buffer, pH 7.5, and dialyzed overnight against 1 l. of 0.02 M Tris buffer, pH 7.5.

Fraction IV—The clear solution, after dialysis, was adjusted slowly to pH 5.3 with ice-cold N acetic acid. The solution was centrifuged at once and the mud-colored precipitate discarded. The supernatant was adjusted immediately to pH 7.5 by addition of cold N potassium hydroxide. Sufficient M Tris buffer, pH 7.5, was added so that the final buffer concentration was 0.12 M. To the solution (60 per cent saturation) was added powdered ammonium sulfate (5.4 gm. to 15 ml.). The mixture was centrifuged after 10 minutes. The supernatant was taken to 70 per cent saturation by the further addition of 1.12 gm. of ammonium sulfate. After standing for 2 hours, the mixture was centrifuged and the precipitate dissolved in 4 ml. of 0.1 M Tris buffer, pH 7.5, and dialyzed overnight against 500 ml. of 0.02 M Tris buffer, pH 7.5.

Fraction V—Usually two batches of enzyme Fraction IV were combined for the following ethanol fractionation. To 6.2 ml. of enzyme Fraction IV was added 0.7 ml. of 0.4 M magnesium chloride. The solution was placed in an ice-salt bath and 1.5 ml. of absolute ethanol at −15° were added slowly, with stirring.[4] The mixture was centrifuged at −10° for 10 minutes at 10,000 × *g*. The precipitate was dissolved in 2 ml. of 0.02 M Tris buffer (Fraction V_a). An additional 1.45 ml. of ethanol were added to the supernatant, and this precipitate was collected and taken up in the same way (Fraction V_b). This last fraction contained the most highly purified enzyme. The supernatant was discarded, and the two active fractions were dialyzed as usual. Fraction V_a was saved, recombined with other batches, and fractionated with ethanol again for a further recovery of highly purified enzyme. The various enzyme fractions were stored at −15°.

RESULTS

Enzyme Purification—As shown in Table I, an approximately 400-fold purification was obtained with a yield of 21 per cent. This is actually a low estimate of the yield, since refractionation of Fraction V_a yields more enzyme of high specific activity. A key step is the retention of the enzyme on the gel during the 0.2 M phosphate elution and its removal with 2 M phosphate. At least a 30-fold purification, with the elimination of other activating enzymes, results from this step. Tyrosine-activating enzyme from guinea pig liver has similar properties (13). The first ammonium sulfate step serves to concentrate the enzyme and also to remove residual heme proteins. The final specific activity has varied from 25 to 32 in various preparations. The enzyme is stable at neutral pH and can be frozen and thawed repeatedly over a period of 2 months with little loss in activity. This is in contrast to the tryptophan-activating enzyme of beef pancreas (7), and the lability of most guinea pig liver-activating enzymes,

[4] The temperature should be kept between −8° and −10° during the alcohol fractionation.

TABLE I

Purification of tyrosine-activating enzyme

Fraction	Volume	Protein content	Total activity*	Specific activity		
				Yield	Hydroxamide	P-P exchange
	ml.	*mg./ml.*	*units*	*%*	*units/mg.*	*μmoles/hr./mg.*
I. Acetone powder extract	220	52.0	935	100	0.082	
II. Calcium phosphate gel eluate	180	0.84	613	67	3.62	40.0
III. 1st ammonium sulfate precipitate	14.5	8.6	520	56	4.15	
IV. 2nd ammonium sulfate precipitate	5.8	6.1	435	47	12.3	138.0
V_b. Ethanol precipitate	1.5	4.4	198	21	30.0	310.0

* In the hydroxamide assay, no enzyme activity was observed without added tyrosine, except with Fraction I. The data for this fraction were obtained by removing residual tyrosine as described previously (5). In the P-P exchange assay, the enzyme activity without added tyrosine was less than 5 per cent of the complete system with Fractions IV and V and varied from 10 to 15 per cent with Fraction II. These blanks have not been subtracted from the values of the last column.

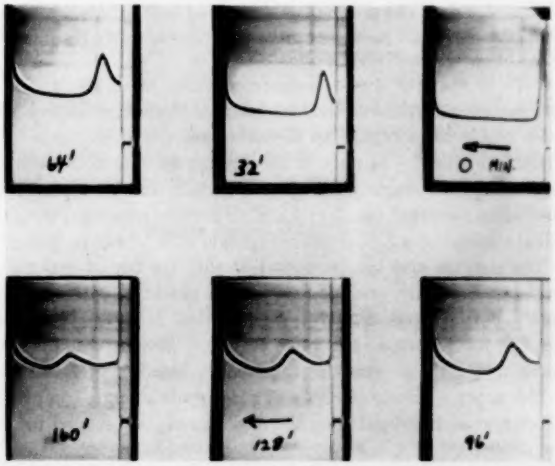

FIG. 1. Ultracentrifuge patterns of tyrosine-activating enzyme. The photographs show enzyme of specific activity = 30 at the times indicated up to 160 minutes, moving from right to left. The conditions were: temperature, 2.7°; enzyme concentration, 4 mg. per ml. in 0.2 M Tris buffer, pH 7.5; rotor speed, 59,780 r.p.m.

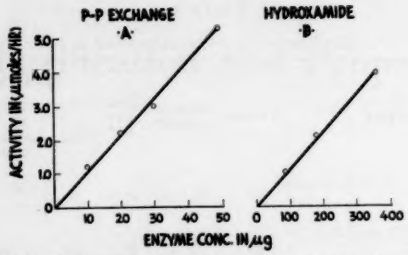

FIG. 2. Effect of enzyme concentration on activity. Standard assay conditions were used (see "Experimental") and the data are given in micromoles per reaction tube. The P-P exchange results (*A*) were calculated from the usual 10-minute assay with the use of the amounts of enzyme shown. Enzyme of specific activity = 11.0 was used in both assays.

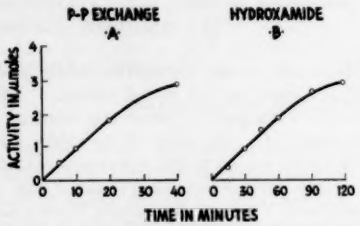

FIG. 3. Time course of tyrosine activation. Standard assay conditions were used. For the P-P exchange, 40 μg. of enzyme of specific activity = 15.2 were used; for hydroxamide formation, 135 μg. of the same preparation.

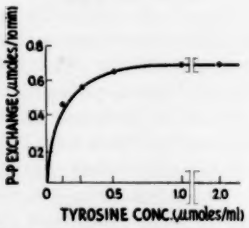

FIG. 4. Effect of tyrosine concentration on the rate of P-P exchange. Standard assay conditions were used with 15 μg. of enzyme of specific activity = 28.0.

although the tyrosine-activating enzyme from this tissue is also more stable to storage than the other activating enzymes.[5]

Enzyme Purity—Studies in the Spinco analytical ultracentrifuge[6] showed a single peak (Fig. 1), which moved with a sedimentation constant ($s_{20,w}$) of 4.2 S. Enzyme recovered from the cell at the end of a 3-hour centrifugation at 2.7° was fully active.

The enzyme also has been studied with the use of paper electrophoresis. Only one component was observed with the most highly purified material when used in 0.02 M phosphate buffer, pH 7.5, for 12 hours at 5 volts per cm. The enzyme does not move under these conditions, probably because of adsorption to the paper. Electrophoresis at pH 9 gave a single component which spread rapidly during the experiment, and moved toward the anode.

[5] E. H. Allen and R. S. Schweet, in preparation.

[6] The authors are indebted to Dr. Jerome Vinograd and Dr. Paul Ts'o, California Institute of Technology, for these analytical ultracentrifuge studies.

No P-P exchange in the absence of amino acids, or with amino acids other than tyrosine, was detected. Adenylic kinase, ATP-hydrolyzing enzymes, and pyrophosphatases were not detected at enzyme concentrations used in the assay. However, ribonuclease was present even in the best preparations. The 280:260 mμ absorption ratio was 1.7, suggesting that no nucleotide-containing material was present.

Enzyme Characteristics—The enzymatic activity was proportional to protein concentration and linear with time under the standard assay conditions (Figs. 2 and 3). In the hydroxamide assay, the rate decrease after 1 hour is the result of inhibition by P-P, a product of the reaction.

Figs. 4–6 show curves of reaction rate with increasing concentrations of each of the three substrates in the P-P exchange reaction. Straight line plots were obtained by the Lineweaver-Burk method (14) and the following K_s values calculated: tyrosine, 6×10^{-5} M; P-P, 3×10^{-4} M; ATP, 5×10^{-3} M. The optimal Mg ion concentration was equimolar with the ATP concentration at all ATP levels. The Mg ion concentrations were the same as the ATP concentrations in the experiments shown in Fig. 5.

The insensitivity of the hydroxamide assay does not permit accurate determination of Michaelis constants. However, for ATP and tyrosine it seems that the results would be similar to the P-P exchange. For example, the rate of hydroxamide formation decreased at ATP concentrations below 0.01 M. The affinity for tyrosine was much higher, since an amount of enzyme which formed 2 μmoles of hydroxamide per hour in the standard assay with a saturating concentration of tyrosine, formed 1.83 μmoles of hydroxamide in 1 hour, when the initial amount of tyrosine was only 2 μmoles. The rate of the hydroxamide reaction increased linearly with hydroxylamine concentration up to about 1.0 M. This concentration has been chosen for the standard assay, although higher levels give somewhat greater activity. Hydroxamide formation was inhibited by P-P, as previously reported (5, 7). The addition of 1.0 μmole of P-P per ml., initially, inhibited hydroxamide formation 28 per cent. The P-P

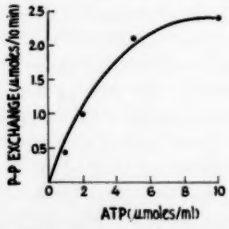

FIG. 5. Effect of ATP concentration on the rate of P-P exchange. Standard assay conditions were used with 51 μg. of enzyme of specific activity = 28.0. The Mg ion concentration was equimolar with the ATP concentration in these experiments.

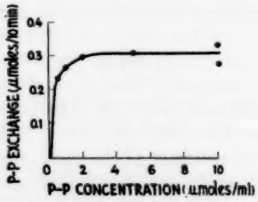

FIG. 6. Effect of P-P concentration on the rate of P-P exchange. Standard assay conditions were used with 7.5 μg. of enzyme of specific activity = 25.0.

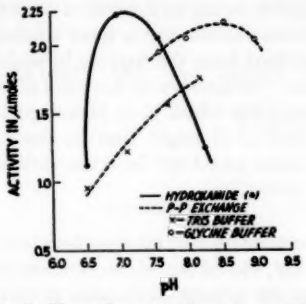

FIG. 7. Effect of pH on the rate of enzyme activity. Standard assay conditions were used and the data are given as micromoles per hour. For the hydroxamide assay (——), 90 μg. of enzyme of specific activity = 25.0 were used. The P-P exchange results (- - -) were calculated from the standard 10-minute assay with 9.0 μg. of the same enzyme.

inhibition here is considerably less than with the tryptophan-activating enzyme studied by Davie *et al.* (7).

The pH optimum for maximal hydroxamide formation is at 7.3, whereas the maximal P-P exchange rate was obtained at pH 8.5 (Fig. 7). Glycine buffer was employed in the standard P-P assay because 0.1 M Tris gave 10 to 15 per cent lower P-P exchange (Fig. 7).

Substrate Specificity—Tyrosine analogues have been tested for activity with the purified enzyme and the following compounds were inactive in both assays when tested at the same concentration[7] as tyrosine: 15 other common amino acids,[8] D-tyrosine, 2,5-dihydroxyphenylalanine, 3,4-dihydroxyphenylalanine, 3-aminotyrosine, 3-nitrotyrosine, *p*-fluorophenylalanine, *o*-tyrosine, *m*-tyrosine, *N*-chloroacetyltyrosine, 3,5-diiodotyrosine, and tyrosine amide. The only compound tested which acted as a substrate was L-3-fluorotyrosine.[9] Even this compound, which resembles tyrosine so closely, was not as effective as tyrosine. In the hydroxamide assay (Table II), fluorotyrosine approached the maximal rate obtained with tyrosine, but only at higher concentrations. However, in the P-P exchange assay, fluorotyrosine gave only 50 per cent of the maximal rate with tyrosine. It seems from these results that almost any substitution on the benzene ring yields an inactive compound. Further, the free amino group in the L-configuration seems necessary for activity. However, it would be of interest to test *p*-hydroxyphenyllactic acid and *p*-hydroxyphenylserine in connection with side-chain specificity.

Effects of Inhibitors—None of the compounds listed above showed any appreciable inhibition at the same concentration as tyrosine, except tyrosine amide. This compound inhibited hydroxamide formation by 30 per cent. Tyramine, however, which cannot be a substrate, was the best inhibitor of the compounds tested. Tyramine inhibited P-P exchange 80 per cent at a concentration of 2×10^{-4} M and hydroxamide formation 50 per cent at 6×10^{-4} M. If the tyrosine concentration of the standard assay was doubled, the inhibition decreased to 50 and 35 per cent, respectively. Phenylethylamine did not inhibit either reaction. The structural requirements for inhibitors which are substrate analogues, also seem to show a high degree of specificity.

In contrast to P-P, phosphate at 0.01 M final concentration was not inhibitory in either assay, and at 0.1 M, inhibited hydroxamide formation 30 to 35 per cent and P-P exchange 20 per cent. Chloramphenicol did not inhibit at 0.01 M. The P-P exchange reaction was inhibited 28 per cent by 5×10^{-4} M

TABLE II

Comparison of tyrosine and fluorotyrosine as enzyme substrates

Each substrate was assayed in the standard way at the indicated concentrations.

Concentration of substrate	Hydroxamide formed			P-P exchange		
	Tyrosine	Fluorotyrosine	Ratio*	Tyrosine	Fluorotyrosine	Ratio*
μmoles/ml.	μmoles/hr.	μmoles/hr.		μmoles/10 min.	μmoles/10 min.	
0.5	—	—	—	1.16	0.46	0.40
1.0	2.1	1.5	0.71	1.20	0.49	0.41
1.6	2.1	1.8	0.86	—	—	—
2.0	—	—	—	1.20	0.58	0.48
3.3	2.1	2.0	0.95	—	—	—

* The ratio is the activity with fluorotyrosine divided by the activity with tyrosine at the same concentration. The dashes indicate that experiments with these concentrations were not done.

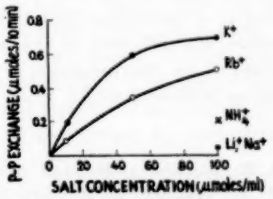

FIG. 8. Effect of cation concentration on the rate of P-P exchange. The ATP, P-P, and tyrosine were used as the Tris salt (see "Experimental"), and the cation was added as the chloride in the indicated amount. Other conditions were standard, with the use of 15 μg. of enzyme of specific activity = 25.0.

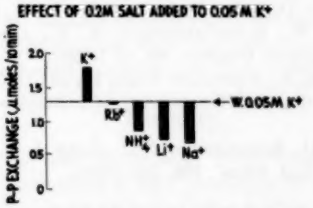

FIG. 9. Effect of extra salt on the rate of P-P exchange. The reaction mixture was the same as given in Fig. 8 with 0.05 M KCl added in all tubes. An additional amount of the appropriate chloride was added as shown by the bars. Other conditions were standard, with the use of 40 μg. of enzyme of specific activity = 20.0.

[7] These compounds, unless noted, were the DL-compounds and were tested at a 0.02 M final concentration. The dihydroxy-compounds, such as 2,5-dihydroxyphenylalanine, and also 3-aminotyrosine and 3-nitrotyrosine gave nonenzymatic color formation in the standard hydroxamide assay and could not be tested with this assay.

[8] The L-amino acids tested were alanine, arginine, glycine, histidine, hydroxyproline, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, and valine.

[9] This compound was a gift from Dr. Carl Niemann, California Institute of Technology.

p-chloromercuribenzoate. Tryptophan-activating enzyme (7) and activating enzymes from guinea pig liver are more sensitive to inhibition by this reagent.[5] The relative insensitivity of the tyrosine enzyme from pancreas may be related to its stability to storage, since the most stable activating enzymes from guinea pig liver (tyrosine and threonine) are also inhibited the least by —SH inhibitors.[5]

Requirements for Cations—No enzyme activity was observed in the absence of added Mg ion. Of more interest is the requirement for K ion shown in Fig. 8 for the P-P exchange reaction. The K ion requirement for the hydroxamide reaction was observed also when salt-free hydroxylamine (see "Experimental") was used (5). No such requirement has been found for other activating enzymes (5). Although Rb ion (Fig. 8) is about 65 per cent as active as K ion at low concentrations, the superiority of K ion is shown at higher salt concentrations. When 0.2 M salt is added to a system containing a suboptimal amount of K ion (0.05 M), only extra K ion stimulates (Fig. 9). Other monovalent cations either have no effect or are inhibitory.

Relationship of P-P Exchange and Hydroxamide Formation—The two activities, P-P exchange and hydroxamide formation, are probably catalyzed by the same enzyme. This is indicated by the substrate specificity, salt requirement and other properties of both reactions, and by the constant activity ratio of 10:1 in favor of P-P exchange shown at various stages of purification (Table I). This ratio varies widely for different enzymes and seems to be a characteristic of the particular amino acid-activating enzyme studied (2, 3, 5, 7). The two reactions, however, differ quantitatively in many ways. In addition to the much smaller amount of P-P than of hydroxylamine needed for obtaining maximal reaction rates, the two reactions differ in their pH optima, the effectiveness of 3-fluorotyrosine as substrate and tyramine as inhibitor, and in their sensitivity to excess salt (5). The available evidence suggests that enzyme-bound, amino acid-AMP (see Equation 1) is the intermediate in both reactions (2, 15, 16) and, therefore, the difference in the two reactions may reside in the reaction of the enzyme-amino acyl-AMP intermediate with P-P *versus* hydroxylamine. It is possible that hydroxamide formation occurs as a result of a nonenzymatic reaction between hydroxylamine and a small amount of free amino acid-AMP dissociated from the enzyme by high concentrations of hydroxylamine. Differences in the ratio of P-P exchange to hydroxamide formation which have been reported for different activating enzymes (2–5) might then be due to differences in dissociation of amino acid-AMP from the various enzymes.

DISCUSSION

The tyrosine-activating enzyme described here, because of its purity and stability, should provide an excellent tool for the more detailed study of the general mechanism of carboxyl-group activation. The nature of the active groups at the enzyme site, the chemistry of the amino acid-AMP-enzyme complex, and the equilibrium constant, are problems which can be studied with the purified enzyme. A comparison of the differences among the various amino acid-activating enzymes would also be of interest. For example, this enzyme contains little or no nucleotide-containing material, in contrast to the tryptophan-activating enzyme (7). This finding also indicates that the reactions studied here do not require the presence of ribonucleic acid, in contrast to the recent report of the participation of ribonucleic acid in alanine activation (17). An estimate of the turnover number of the P-P exchange reaction can be made from the sedimentation constant. This value is very approximately 400 moles per mole of enzyme per minute, which is lower than that for many enzymatic reactions.

SUMMARY

Procedures for the isolation of a tyrosine-activating enzyme are described. The physical and enzymatic criteria cited indicate that the best preparations are nearly pure. The only compound which served as a substrate, in addition to tyrosine, was 3-fluorotyrosine. Tyramine was the best inhibitor among the compounds tested. Two cations, K and Mg, were required for enzymatic activity. Differences in the formation of tyrosine hydroxamide and pyrophosphate exchange are discussed in relation to the mechanism of the reaction.

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Isolation of D- and L-Glutamyl Polypeptides from Culture Filtrates of *Bacillus subtilis*

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(Received for publication, May 19, 1958)

When grown under appropriate conditions certain strains of *Bacillus subtilis* produce γ-glutamyl polypeptide which can be isolated from culture filtrates (1, 2). *Bacillus anthracis* also produces a γ-glutamyl polypeptide which is present in the capsule of the organism. Although it is generally agreed that the peptide from *B. anthracis* is composed mainly, if not entirely, of the D-isomer of glutamic acid (3, 4), preparations from *B. subtilis* have been reported to contain both isomers in various proportions (1, 2, 5). A recent report from this laboratory (6) dealt with the effect of concentration of Mn^{++} on the configuration of glutamic acid in polypeptide produced by *B. subtilis*. With a high concentration of Mn^{++} in the growth medium the glutamic acid in the peptide was composed of 80 per cent or more of the D-isomer, and with a low concentration of Mn^{++} the proportion of the D-isomer in the peptide was about 40 per cent. Further work resulted in the isolation of peptides composed predominantly, if not entirely, of either L- or D-glutamic acid. This report describes the isolation of these peptides and their solubility characteristics.

EXPERIMENTAL

Materials and Methods

Production of Glutamyl Polypeptides—The medium and method for production of glutamyl polypeptide by *B. subtilis* (ATCC 9945A) have been described elsewhere (2, 6). For the preparation of peptide from *B. anthracis*, the virulent strain M-36 was grown in covered stainless steel trays (16 x 12 x 1 inch) containing 600 ml. of the following medium: Difco nutrient broth, 8 gm.; Difco yeast extract, 3 gm.; glucose, 1 gm.; $NaHCO_3$, 5 gm.; agar, 20 gm.; and distilled water, to 1 l. The trays were inoculated with a spore suspension and incubated at 37° for 48 hours in an atmosphere of 70 per cent air and 30 per cent CO_2. The growth was scraped from the agar surface and autoclaved for 30 minutes at 121°. The extracted peptide was isolated by precipitation with ethanol followed by reprecipitation as the silver salt according to the method of Hanby and Rydon (4). The free acid peptide was obtained by precipitating the silver with excess HCl, and dialyzing the supernatant solution against distilled water at 4° until free of chloride. The dialyzed peptide was lyophilized. Paper chromatography showed that no free amino acids were present in solutions of the peptide and that only glutamic acid was released upon acid hydrolysis. As determined with L-glutamic acid decarboxylase, 1.4 per cent of the glutamic acid in acid hydrolysates of the peptide was the L-isomer.

$(C_5H_7O_3N)_n$

Calculated: N 10.85, glutamic acid released on hydrolysis, 113.9

Found: N 10.95, glutamic acid released on hydrolysis, 112.8

Labeled Glutamic Acid—L-Glutamic acid uniformly labeled with C^{14} was purchased from Nuclear-Chicago Corporation, Chicago, Illinois.

Analytical Methods—Total N was determined by the micromethod of Johnson (7). Total glutamic acid was determined by the paper chromatographic method of Housewright and Thorne (8). L-Glutamic acid was assayed manometrically with L-glutamic acid decarboxylase by the method of Umbreit and Gunsalus (9), except that cell-free extracts of *Escherichia coli* were used. D-Glutamic acid was determined manometrically with D-glutamic acid oxidase from *Aspergillus ustus* (10) or estimated indirectly by substracting the amount of the L-isomer from the total amount. Peptide was determined as glutamic acid released upon hydrolyzing the peptide with 3 N HCl by autoclaving at 121° for 2 hours. Peptide in culture filtrates was determined by precipitating it as the copper salt by addition of excess saturated $CuSO_4$. The precipitate was collected by centrifugation and hydrolyzed for determination as glutamic acid as described.

Radioactivity Measurements—For determination of total radioactivity in peptide or glutamic acid, samples were converted to CO_2 by oxidation with potassium persulfate (11). Carbon dioxide from the α-COOH groups of total glutamic acid was obtained by decarboxylation with ninhydrin, and CO_2 from the α-COOH of L-glutamic acid was released with L-glutamic acid decarboxylase in cell-free extracts of *E. coli*. The CO_2 was collected as $BaCO_3$ which was plated on aluminum disks and counted in a windowless, flow type Geiger counter. Specific activities are reported as counts per minute per mg. of $BaCO_3$.

RESULTS

Isolation of D- and DL-Peptides from Culture Filtrate—To obtain peptide containing a high percentage of D-glutamic acid, *B. subtilis* was grown for 90 hours in the synthetic Medium E (6) with 6.15×10^{-4} M $MnSO_4$. Most of the cells were removed by Sharples centrifugation. However the culture was very viscous and some cells remained in the centrifuged solution. Analysis of an aliquot by the copper precipitation method showed that

TABLE I

Analysis of peptides isolated from cultures of Bacillus subtilis

Preparation	$MnSO_4$ in medium	Form of peptide	Nitrogen	Glutamic acid*		
				Total	L-Isomer	D-Isomer
	moles/l.		%	%	*% of total*	*% of total*
Culture filtrate A	6.15×10^{-4}	Total copper precipitate			20.0	
A-I		Free acid	11.2	115.4	46.1	54.6
A-II		Sodium salt	9.3	99.8	8.8	94.0
Culture filtrate B	1.54×10^{-7}	Total copper precipitate			57.0	
B-I		Free acid	11.3	114.1	49.2	49.2
B-II		Sodium salt	9.5	91.0	90.0	7.0
Theoretical		Free acid $(C_5H_7O_3N)_n$	10.85	113.9		
		Sodium salt $(C_5H_6O_3N\ Na)_n$	9.27	97.4		

* The D-isomer was determined with D-glutamic acid oxidase and the L-isomer was determined with L-glutamic acid decarboxylase. With all the preparations glutamic acid was the only amino acid detected after acid hydrolysis.

the solution contained about 17 mg. of peptide per ml. and that about 80 per cent of the glutamic acid released on acid hydrolysis was the D-isomer. The peptide in 3200 ml. of such culture fluid was precipitated by adding 400 ml. of saturated $CuSO_4$ solution. The supernatant solution was discarded and the gummy precipitate was added to about 500 ml. of 1 N HCl. The copper peptide dissolved slowly and even before it was all in solution some of the peptide began to precipitate as a white, granular material. This precipitation was hastened by allowing the mixture to stand in a cold room at $-10°$ for about 3 hours. The precipitated peptide (as free acid) was collected on a sintered glass filter (coarse porosity). The free acid was insoluble in water but upon the addition of sufficient NaOH to form the sodium salt it dissolved readily in about 300 ml. of water. This solution was centrifuged at $12,000 \times g$ for 30 minutes to remove some insoluble material including the cells that were not removed from the original culture fluid. The peptide was reprecipitated by adding sufficient HCl to give a 1 M solution and placing it in the cold. The precipitate was collected as before and this procedure was repeated a second time. The final precipitate of peptide in the free acid form was dried in a vacuum oven at 50° for 24 hours. It should be pointed out that in these experiments no attempt was made to isolate this acid-insoluble peptide quantitatively. The yield (Preparation A-I, Table I) was 6 gm. of peptide composed of about 50 per cent of each isomer of glutamic acid.

To the original supernatant solution containing the copper and acid-soluble peptide was added a concentrated solution of Na_2S until a precipitate no longer formed. The pH was adjusted to 6.0 with NaOH, and CuS was removed by centrifugation. The supernatant solution was adjusted to pH 8.0 and the peptide was precipitated by pouring into 4 volumes of ethanol. The precipitate was dissolved in about 300 ml. of water and the solution was stirred for 30 minutes with 2 gm. of Norit A (American Norit Company) and then adjusted to pH 3 to 4 with HCl to reduce the viscosity. The Norit was removed by centrifuging at $12,000 \times g$ followed by filtration through a Mandler filter to remove the last traces. The solution was dialyzed for 20 hours against distilled water at 4° and finally adjusted to pH 7.0 with NaOH and then lyophilized. The yield was 20 gm. (Preparation A-II, Table I) of peptide, the glutamic acid of which was composed of 94 per cent of the D-isomer. Analyses of Preparations A-I and A-II are given in Table I.

Isolation of L- and DL-Peptides from Culture Filtrate—To obtain peptide containing a high percentage of L-glutamic acid, *B. subtilis* was grown for 90 hours in Medium E containing 1.54×10^{-7} M $MnSO_4$, and the cells were removed as above. Analysis of the culture fluid (3000 ml.) revealed that the solution contained about 18 mg. of peptide per ml. and that 57 per cent of the glutamic acid released on acid hydrolysis was the L-isomer. The peptide was precipitated by the addition of 4 volumes of ethanol and the precipitate was taken up in about 500 ml. of water. Centrifugation at $12,000 \times g$ removed some insoluble material. HCl was added to a final concentration of 1 N and the solution was held in a room at $-10°$ for 3 hours. The precipitate of DL-peptide was collected on a sintered glass filter and washed with water. It was dissolved in water by adding NaOH to form the sodium salt and reprecipitated by acidifying with HCl. The final precipitate was dried in a vacuum oven at 50° for 24 hours. The yield was 43 gm. of peptide (Preparation B-I, Table I) which was composed of about 50 per cent of each isomer of glutamic acid.

The supernatant solution from the first precipitation of the DL-peptide was adjusted to pH 5 with NaOH and the solution was dialyzed against distilled water for 24 hours at 4°. The dialyzed solution was adjusted to pH 7.0 and lyophilized. The yield was 3 gm. of peptide (Preparation B-II, Table I) the glutamic acid of which was composed of about 90 per cent of the L-isomer. Analyses of Preparations B-I and B-II are given in Table I.

Each of the two isolation procedures described could be used for separating either the D- or L-peptide from the DL-peptide.

Coprecipitation of D- and L-Peptides—In the experiments described above in which D-, L- and DL-peptides were isolated, it appeared that D- and L-peptides might be coprecipitating stoichiometrically from acid solutions to give preparations composed of 50 per cent of each isomer and that the isomer which occurred in excess remained in the supernatant solution. Thus in filtrates from cultures grown with a high concentration of Mn^{++} the D-peptide was in excess and with a low concentration of Mn^{++} the L-peptide was in excess.

Experiments were carried out on the solubilities of the various peptides. All of the peptides were soluble in neutral solution. In acid solution the D- and L-peptides were each soluble but the DL-peptide was insoluble. When solutions of the D- and L-peptides in 1 N HCl were mixed, a precipitate formed which was composed of about 50 per cent of each isomer. If the two peptides were mixed in equal proportions, they precipitated almost quantitatively from solution. If they were mixed in unequal proportions the one in lower concentration precipitated almost quantitatively with an equal amount of the other, leaving the excess in the supernatant.

Some experimental results are shown in Table II. Neutral stock solutions of the D-, L- and DL-peptides from *B. subtilis* and the D-peptide from *B. anthracis* were prepared, and appropriate aliquots were combined to give the mixtures listed in the table. The volumes were made up to about 5 ml. with water.

Sufficient 10 N HCl was added to each to give a final concentration of about 1 N and the mixtures were held at 4° overnight. The precipitates were removed by centrifugation and washed once with water, and were then dissolved in water by the addition of NaOH and analyzed. No attempt was made to recover the peptides quantitatively. In this and similar experiments the L-peptide of *B. subtilis* coprecipitated from acid solution with both the D-peptide from *B. subtilis* and the D-peptide from *B. anthracis*. Either peptide alone was very soluble in acid solution. The peptide from *B. anthracis* did not precipitate with the D-peptide from *B. subtilis*.

Some other compounds were tested for their ability to coprecipitate with the D- or L-peptide from acid solution. Among them were α-D-glutamyl-D-glutamic acid, γ-D-glutamyl-D-glutamic acid, α-L-glutamyl-L-glutamic acid, glutathione, poly-α-L-glutamic acid and gelatin. Gelatin was the only one of these compounds that coprecipitated with the peptides, but this precipitation was not stereospecific. Both the D- and L-peptides precipitated with gelatin from acid solution but not from neutral solution.

The above results suggested that the *B. subtilis* peptides previously isolated from acid solutions and which were always composed of nearly 50 per cent of each isomer, were in fact mixtures of two peptides, one composed mostly, if not entirely, of the L-isomer and one similarly composed of the D-isomer. Further evidence for this was obtained in an experiment with a C^{14}-labeled peptide preparation described below.

Preparation of C^{14}-Labeled Peptide—The procedure was the same as that described previously for preparing DL-peptide (2) except that the L-glutamic acid used in the medium (20 mg. per ml.) was uniformly labeled with C^{14}. The culture filtrate obtained from 150 ml. of medium yielded 1.66 gm. of peptide in the free acid form. The C^{14}-L-glutamic acid added to the medium had a specific acitivity of 770. The peptide had a specific activity of 357 and the glutamic acid released on acid hydrolysis was composed of 49.4 per cent of the L-isomer, as determined with L-glutamic acid decarboxylase and 50.6 per cent of the D-isomer, as estimated by difference. The specific activity of the α-COOH carbon from the total glutamic acid was 372 and that of the α-COOH carbon from the L-isomer was 380. These data indicated that the peptide was uniformly labeled.

Proof that C^{14}-DL-Peptide Was Mixture of D- and L-Peptides—If the radioactive DL-peptide were a mixture of individual D- and L-peptides, after mixing it in neutral solution with unlabeled D- or L-peptide and acidifying, the specific activity of the reprecipitated DL-peptide should be less than it was originally and the excess L- or D-peptide remaining in solution should be labeled. However, if the labeled DL-peptide had D- and L-glutamic acid linked together in the same peptide chain, no change in specific activity should occur upon mixing with unlabeled peptide and reprecipitating.

The results of an experiment performed to determine which of these possibilities occurs are shown in Table III. Sample 1 had 230 mg. of C^{14}-DL-peptide; Sample 2 had 230 mg. of C^{14}-DL-peptide and 105 mg. of unlabeled D-peptide; and Sample 3 had 230 mg. of C^{14}-DL-peptide and 950 mg. of unlabeled D-peptide. The unlabeled D-peptide was Preparation A-II described in Table I. The mixtures were prepared by pipetting appropriate aliquots of neutral stock solutions. The final volume was about 25 ml. after adding 2.5 ml. of 10 N HCl. These solutions were held at 4° for 3 hours. The precipitates were collected by centrifugation, washed 2 times with 0.1 N HCl and once with water, and finally dissolved in water by adding NaOH. The supernatant solutions from the first centrifugation were dialyzed against distilled water for 12 hours at 4° to remove the HCl. No attempt was made to recover quantitatively either the precipitated peptides or those in the supernatant solutions.

The specific activity of the original DL-peptide was 357 and this did not change significantly upon reprecipitation of the pep-

TABLE II

*Coprecipitation of D- and L-peptides**

Mixture No.	Peptides in original solution	Precipitated peptide		
		Total	L-Isomer	D-Isomer
		mg.	mg.	%
1	DL-Peptide, 69 mg.	36.1	18.8	48
2	L-Peptide, 70 mg.	No precipitate		
3	D-Peptide, 70 mg.	No precipitate		
4	Solutions 2 and 3 mixed together	71.8	39.0	46
5	L-Peptide, 11.5 mg. D-Peptide, 11.5 mg.	13.2	7.0	47
6	L-Peptide, 50 mg. D-Peptide, 100 mg.	70.0	35.3	50
7	L-Peptide, 50 mg. D-Peptide (*Bacillus anthracis*), 50 mg.	62.5	31.0	50

* Stock solutions of the sodium salts of the four peptides were prepared in water and aliquots were used to prepare the mixtures described. Final volume was about 5 ml. Sufficient 10 N HCl was added to give a final concentration of about 1 N. No attempt was made to obtain quantitative recovery of the precipitated peptides. The DL-peptide was Preparation B-I, the L-peptide was Preparation B-II and the D-peptide (*Bacillus subtilis*) was Preparation A-II of Table I. The peptide from *B. anthracis* was the preparation described under "Experimental." Amounts of peptide are given as equivalent amounts of glutamic acid.

TABLE III

*Equilibration of unlabeled D-peptide with C^{14}-D-peptide in uniformly labeled DL-mixture**

No.	Original solution: Peptides	Acid-insoluble peptide					Acid-soluble peptide				
			D-isomer	Specific activity				D-isomer	Specific activity		
				Found	Theoretical				Found	Theoretical	
		mg.	mg.	%		%†	%†	mg.	%		%†	%†
1	C^{14}-DL, 230	177	48	359	100	100	trace					
2	C^{14}-DL, 230 C^{12}-D, 105	204	51	272	76	76	61	92	183	51	52
3	C^{14}-DL, 230 C^{12}-D, 950	232	56	200	56	55	695	92	37	10	11

* Amounts of peptide are given as equivalent weights of glutamic acid. Specific activities are given as counts per minute per mg. of $BaCO_3$. The D-peptide was Preparation A-II of Table I. No attempt was made to recover the peptides quantitatively.

† Percentage of specific activity of original C^{14}-DL-peptide.

tide (Sample 1). In Sample 2 the ratio of added C^{14}-DL-peptide to unlabeled D-peptide was about 2.2:1 (230 mg.:105 mg.). Assuming that the C^{14}-DL-peptide was a mixture of a D- and an L-peptide, the ratio of C^{14}-D-peptide to unlabeled D-peptide was about 1.1:1 (115 mg.:105 mg.). After the peptides were mixed in solution the specific activity of the total D-peptide should be 52 per cent of the original value. The DL-peptide in the precipitate resulting from acidifying the solution should have a specific activity equal to 76 per cent of the original, and the excess D-peptide remaining in the supernatant solution should be labeled and have a specific activity equal to 52 per cent of that of the original labeled peptide. The experimental results agree with the calculated values.

In Sample 3 the ratio of added C^{14}-DL-peptide to unlabeled D-peptide was about 1:4.1 (230 mg.:950 mg.), and thus the ratio of C^{14}-D-peptide to unlabeled D-peptide was about 1:8.3 (115 mg.:950 mg.). By the same reasoning as above, the specific activity of the reprecipitated peptide should be about 55 per cent of the original value and the excess D-peptide remaining in the supernatant solution should be labeled and have a specific activity equal to 11 per cent of that of the original peptide. Again the experimental results agree with the calculated values.

DISCUSSION

The possibility that a peptide chain composed predominantly of one isomer may contain a small proportion of the other isomer has not been ruled out. The preparations which contain 90 to 95 per cent of one isomer may be contaminated with a small amount of a peptide containing the other isomer or they may, in fact, have small amounts of the second isomer in peptide linkage with the predominant isomer.

The recent report of Volcani and Margalith (12) on an enzyme which quantitatively released L-glutamic acid from glutamyl polypeptide preparations containing both isomers of glutamic acid is further evidence that such preparations are mixtures of two peptides.

SUMMARY

1. By controlling the concentration of Mn^{++} in the medium and taking advantage of the solubility characteristics of the peptides, we isolated in highly purified form from culture filtrates of *Bacillus subtilis* three glutamyl polypeptide preparations: one was composed predominantly, if not entirely, of L-glutamic acid; a second was composed predominantly, if not entirely, of D-glutamic acid; and the third was composed of 50 per cent of each isomer.

2. All three preparations and mixtures of them were soluble in neutral solution. The DL-peptide was insoluble in acid solution. Although D- and L-peptides were each separately soluble in acid solution, when mixed together they coprecipitated. The D-peptide from *B. anthracis* also coprecipitated from acid solution with the L-peptide from *B. subtilis*.

3. Evidence is presented that preparations of polypeptide from *B. subtilis* which contain both isomers of glutamic acid are mixtures of two peptides, one composed predominantly of the D-isomer and the other composed predominantly of the L-isomer. Under our experimental conditions *B. subtilis* probably does not produce a glutamyl polypeptide containing large proportions of both D- and L-glutamic acid in the same peptide chain.

Acknowledgment—We wish to express our appreciation for the excellent technical assistance given by Mr. Raymond Gregoire.

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Purification and Properties of an Inducible β-Glucosidase of Yeast*

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(Received for publication, June 19, 1958)

In the past decade considerable advances have been achieved in the study of induced enzyme biosynthesis in microorganisms. A comparison of three of the better studied systems, β-galactosidase in *Escherichia coli* (1), penicillinase in *Bacillus cereus* (2), and α-glucosidase in *Saccharomyces cerevisiae* (3) indicates that there are a number of differences in these inductions. In order to compare further several inducible systems within the same group of organisms, a search was undertaken for a second inducible system in yeast.

The wide distribution of β-glucosidase among plants (4), fungi (5), and yeasts (6) suggested this enzyme as a possible system for examination. Among the yeasts, the capacity of intact cells to hydrolyze β-glucosides varies both with the strain and conditions of growth (6). For example, *Candida tropicalis* NCYC4 hydrolyzes salicin but not arbutin, whereas several strains of *S. cerevisiae* are active on arbutin but weak on salicin. Since observations of this type can be interpreted as evidence for several β-glucosidases, as has been demonstrated in fungi (7), or stereospecific permeation systems (8), further purification and study of the purified enzymes or enzymes of yeast are indicated. Neuberg and Hofmann (9) have partially purified a β-glucosidase from plasmolyzed yeast by ethanol-diethyl ether precipitation and examined some of its properties. A thermolabile enzyme, which is active against esculin, has been reported in bakers' yeast (10).

The demonstration of an inducible β-glucosidase in *S. cerevisiae* strain Yeast Foam (11) has led us to examine this enzyme further. As a prerequisite to an interpretation of the induction experiments, it was necessary not only to purify the enzyme and examine its specificity but also to provide a basis for quantitative determination of the enzyme content of the organism under various conditions.

EXPERIMENTAL

Organism—A pink isolate of *Saccharomyces cerevisiae* strain Yeast Foam was grown aerobically at 30° in a synthetic medium (3) with succinate as carbon source and 0.01 M methyl-β-D glucoside as the inducer of β-glucosidase.

Analytical—In this study of enzyme purification and properties, β-glucosidase activity was usually estimated by spectrophotometric measurement of the continuous release of *p*-nitrophenol from NPG.[1]

0.1 ml. of a proper dilution of the enzyme preparation was added to 2.9 ml. of a preincubated (10 minutes, 30°) reaction mixture that contained 2.5 ml. of 0.067 M potassium phosphate buffer, pH 6.8, 0.3 ml. of 10^{-2} M NPG, and 0.1 ml. of reduced glutathione (2.5 mg. per ml.). Optical density of the mixture was determined periodically at 400 mμ by using a Beckman Spectronic "20" colorimeter. In cases in which a discontinuous assay was necessary the Beckman model DU spectrophotometer was used. A unit of enzyme is defined as that amount of enzyme necessary to bring about an optical density change of 0.001 per minute per 3 ml. of reaction mixture. *p*-Nitrophenol was determined by the absorption at its maximum (400 mμ) either in the continuous assay, pH 6.8 ($E^{M}_{1\,cm.}$ 9.6×10^3), or where indicated in the discontinuous assay in 0.1 M Na_2CO_3, pH 10.2 ($E^{M}_{1\,cm.}$ 1.83×10^4).

The rates of hydrolysis of other glucosides were determined by estimating the rate of glucose release with a coupled hexokinase and glucose-6-phosphate dehydrogenase system or in some cases manometrically with *Torula monosa* (3). Hexokinase and glucose-6-phosphate dehydrogenase were partially purified from *T. monosa* by the method of Kunitz and McDonald (12). Separate hexokinase and glucose-6-phosphate dehydrogenase preparations devoid of 6-phosphogluconate dehydrogenase were obtained by chromatography on DEAE-cellulose (13) columns.

Protein was determined routinely by the colorimetric method of Lowry *et al.* (14) with the Folin-Ciocalteu reagent and with crystalline serum albumin as the standard.

Reagents—The following compounds were synthesized as indicated: methyl-β-D-glucopyranoside (15), *p*-nitrophenyl-β-D-glucopyranoside (16), and *p*-nitrophenyl-α-D-glucopyranoside (17). Phenyl-β-D-glucopyranoside, phenyl-thio-β-D-glucopyranoside, phenyl-thio-β-D-galactopyranoside, arbutin, salicin, esculin, amygdalin, and *o*-nitrophenyl-β-D-galactopyranoside were gifts of Dr. J. Monod. Pure D-xylose was obtained from H. G. Fletcher, Jr. Methyl-β-xyloside, D-mannose, D-galactose, D-glucuronic acid, D-gluconic acid, L-arabinose, and D-ribose were purchased from Nutritional Biochemical Corporation, and cellobiose from Difco Laboratories. All other glucosides used were kindly furnished by Dr. S. Spiegelman. Mercaptoethanol was purchased from the Matheson Company, DEAE-

* This investigation was supported in part by a research grant (E1459) from the Division of Research Grants of the National Institutes of Health, United States Public Health Service. A preliminary report was presented at the meeting of the Society of American Bacteriologists, Chicago, Illinois, May, 1958.

[1] The abbreviations used are: NPG, *p*-nitrophenyl-β-D-glucopyranoside; PVP, polyvinylpyrrolidone; Tris, tris(hydroxymethyl)aminomethane; CMB, *p*-chloromercuribenzoate.

cellulose No. 40 from the Brown Company, and PVP from the General Aniline and Film Corporation. The latter was dialyzed against water and concentrated by lyophilization to a 20 per cent solution before use.

Deionized, glass-distilled water was used for all experiments.

Purification of β-Glucosidase—Unless otherwise indicated, all steps were carried out in the cold room. *S. cerevisiae* strain Yeast Foam was grown in 1-l. Erlenmeyer flasks containing 200 ml. of phosphate-succinate synthetic medium with 10^{-2} M methyl-β-D-glucoside. After the addition of a 5 per cent inoculum, the flasks were shaken at 30° aerobically on a rotary shaker for 16 to 20 hours. The cells were harvested, washed, resuspended in a minimal amount of distilled water, and freeze-dried. The resulting powder was stored at −20° until required. Such a preparation maintained its β-glucosidase activity indefinitely.

Fractions I, II, and III—4 gm. of dried cells were suspended by stirring in 80 ml. of distilled water and centrifuged. The cell paste was resuspended in 80 ml. of distilled water containing 10^{-4} M mercaptoethanol, and placed in a Raytheon 10 kc sonic oscillator with 20 gm. of Superbrite glass beads. The suspension was subjected to maximal sonic oscillation treatment at 1° for 1 hour under a N_2 atmosphere. This treatment was found sufficient to release 95 per cent of the β-glucosidase activity from the cell debris. The suspension (Fraction I, 85 ml.) was centrifuged for 1 hour at 30,000 × *g*. The supernatant solution (Fraction II, 75 ml.) was centrifuged for 1 hour at 81,000 × *g* in a Spinco preparative centrifuge at 2°. The resulting pellet was discarded, and the supernatant was recentrifuged at 144,000 × *g* for 6 hours. Although some activity remained in the supernatant, its specific activity was so low that this fraction was discarded. The pellet was brought into solution with 0.02 M potassium phosphate buffer, pH 6.8 (Fraction III, 15 ml.).

Fraction IV, $Ca_3(PO_4)_2$ Gel Absorption—Fraction III was adjusted to pH 5.6 with dilute HCl. 1 ml. of $Ca_3(PO_4)_2$ gel (60 mg. per ml.) was added to give a gel-protein ratio of 3:1. The suspension, after sitting in the cold 3 to 5 minutes, was centrifuged. The gel pellet is eluted with three quantities of 0.067 M potassium phosphate buffer, pH 6.8 (Fraction IV, 7.7 ml.).

Fraction V, DEAE-Cellulose Chromatography—Fraction IV was concentrated to approximately 2 ml. by dialysis against a 20 per cent aqueous solution of PVP, and then it was dialyzed for 24 hours against 0.01 M Tris and 0.005 M magnesium acetate buffer, pH 7.5. A 25-cm. long DEAE column in a glass column 1 cm. in diameter was prepared with commercial DEAE-cellulose according to the method of Sober *et al.* (18).

The dialyzed fraction was added to the column, and an increasing amount of NaCl gradient was applied by means of two reservoirs; one contained 200 ml. of 1.5 M NaCl, and the other contained 200 ml. of the buffer mentioned in the previous paragraph. A pressure of 1.5 pounds per sq. in. was applied to the whole system and fractions of 1.5-ml. volume were collected. The column was cooled by a refrigerant at 1° which passed through a jacket around the column. Each fraction was analyzed for protein and β-glucosidase activity. The enzyme came off in a fairly sharp peak at Fractions 23 to 27 with some trailing, while a large portion of the protein came off before the enzyme peak (Fig. 1). Those fractions with the highest specific activity (23 to 27) were combined (Fraction V, 4.5 ml.).

Fraction VI, Zone Electrophoresis—Fraction V was concentrated to 2 ml. by dialysis against 20 per cent PVP. This preparation was dialyzed for 24 hours against 0.02 M potassium pyrophosphate and 10^{-4} M mercaptoethanol buffer, pH 8.4, and subjected to starch ionophoresis as previously described (19). The enzyme was usually found between 18 and 20 cm. from the origin (10-cm. mark). This portion of the starch block was cut out and eluted with three portions (approximately 5 ml.) of 1 M potassium phosphate buffer, pH 6.8. This eluate was concentrated by dialysis against 20 per cent PVP, and then was dialyzed against 0.01 M Tris and 0.005 M magnesium acetate buffer, pH 7.5. The resulting fraction (Fraction VI, 40 ml.) was used for this study. Table I summarizes the purification procedure.

Purity of Enzyme—Attempts to purify the crude β-glucosidase by the use of organic solvents and salt precipitation have not proved successful. A degree of purification equivalent to that achieved by the method shown in Table I has been reproduced by a multiple adsorption and elution of the enzyme from $Ca_3(PO_4)_2$ gel (three times), negative adsorption on Cγ

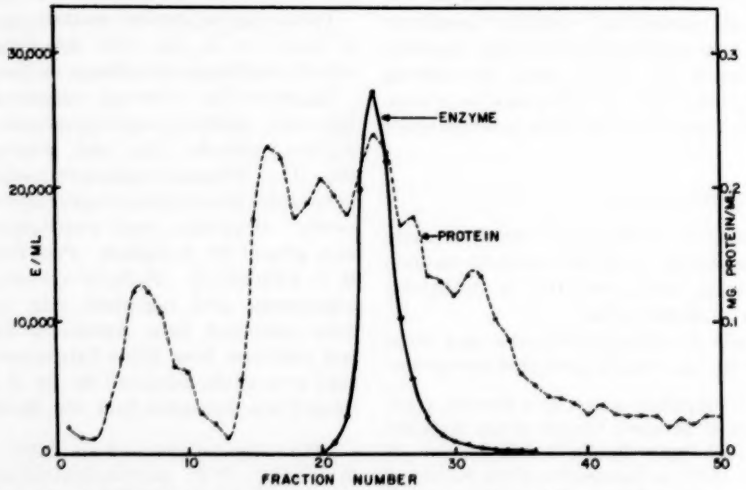


FIG. 1. Chromatography of partially purified β-glucosidase on a DEAE-cellulose column. The fraction was 1.5 ml. The other conditions are described in text. *E* = units of β-glucosidase.

alumina gel, repeated electrophoresis on starch columns (twice), and elution from DEAE-cellulose columns. Treatment of Fraction VI with Cγ alumina gel did not increase the specific activity of the enzyme.

Fraction VI was found to be devoid of hexokinase, glucose, and glucose-6-phosphate and 6-phosphogluconate dehydrogenases. With the appropriate chromogenic substrates the enzyme was also found to be free from contamination with α-glucosidase, α-galactosidase, and β-galactosidase. With the use of the procedure of Aronson (20), Fraction VI was directly examined for transglucosidation activity. A chromatographic examination of reaction mixtures incubated for periods of 2 minutes to 18 hours with phenyl-β-D-glucoside and glucose, fructose, or galactose failed to reveal the accumulation of polysaccharides or other intermediates. Consequently, it seems that the enzyme is reasonably free from contaminating enzymes that would interfere with the specificity studies of β-glucosidase.

The homogeneity of Fraction VI was examined by observing the kinetics of thermal inactivation at 56° in 0.067 M phosphate buffer. The resulting loss of β-glucosidase activity (Fig. 2) followed first order kinetics; this is consistent with the involvement of a single molecular species in the hydrolysis of β-glucosides. A further analysis of the homogeneity of Fraction VI must await isolation of the enzyme on a larger scale.

The molecular weight of β-glucosidase (Fraction VI) was estimated by sedimentation in an analytical Spinco ultracentrifuge. The enzyme had a sedimentation constant (s_{20}) of 11.3 S. Assuming normal protein density and frictional ratios for β-glucosidase, the molecular weight of the enzyme is approximately 300,000.

Kinetics of Enzyme Action

The determinations of *p*-nitrophenol formed during the hydrolysis of NPG indicate that the kinetics obeyed the zero order law over the portion of the curve up through 55 per cent hydrolysis. The initial zero order velocity of NPG hydrolysis is directly proportional to the first power of the enzyme concentration.

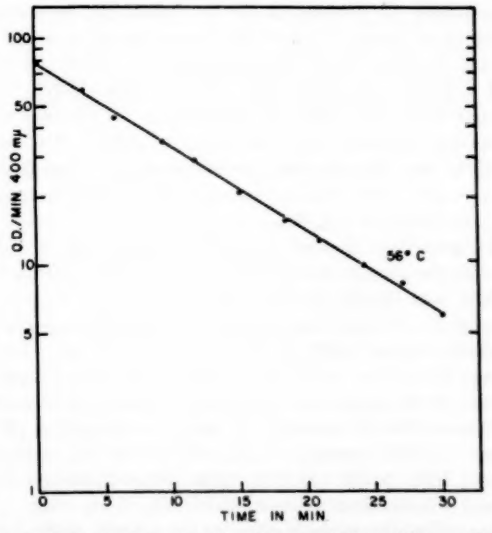


FIG. 2. Temperature inactivation of β-glucosidase. To 1.915 ml. of 0.067 M phosphate at 56° ± 0.1° was added 0.085 ml. of Fraction VI (1530 units). At intervals 0.1 ml. was added to the assay mixture at 30° and assayed as described in the text. *O.D.* = optical density.

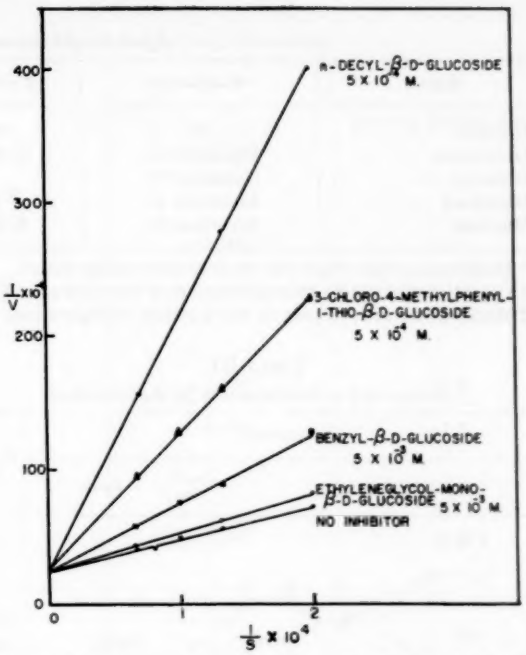


FIG. 3. The competitive inhibition of NPG hydrolysis by several β-glucosides. The reaction mixture (1.0 ml.) contained the indicated concentration of inhibitor and NPG (*S*), 0.067 M phosphate buffer, pH 6.8, and 0.1 ml. (45 units) of a 1:40 dilution of Fraction VI. After 2 minutes the reaction was stopped by the addition of 2.0 ml. of 0.1 M carbonate buffer, pH 10.2, and the mixture was measured as described in "Experimental."

The stoichiometry of the β-glucosidase action was essentially quantitative. For each mole of NPG hydrolyzed, 1 mole of glucose and 1 mole of *p*-nitrophenol are liberated. The hydrolytic nature of the reaction was further indicated by the fact that neither the yield nor the initial rates of hydrolysis were affected by the presence or absence of 0.067 M phosphate. A 35 per cent inhibition of the rate of hydrolysis, however, was observed in the presence of 0.067 M Tris buffer, pH 6.8.

With NPG as substrate, a typical Michaelis-Menten relationship was obtained between the substrate concentration and the initial velocity of the reaction. A Lineweaver-Burk plot

TABLE I

Purification of β-glucosidase

Fraction No.	Total volume	Activity	Protein	Specific activity	Yield
	ml.	*units*/ml.*	*mg./ml.*	*units/mg. protein*	%
I	85	3,800	3.82	1,000	100
II	75	4,100	2.30	1,700	95
III	15	13,000	2.00	6,500	60
IV	7.7	24,800	1.69	14,700	59
V	4.5	22,600	0.226	100,000	31
VI	4.0	18,000	0.149	121,000	22

* 1 unit = 0.001 change in optical density (400 mμ) per minute.

TABLE II

Affinities of hexoses and pentoses for β-glucosidase

Hexoses	Modification*	K_i† × 10^5	Pentoses	Modification‡	K_i† × 10^5
D-Glucose	0	850	D-Xylose	0	10,500
D-Glucuronic	Oxidation C_6	10,500	D-Ribose	Inversion C_3	0
D-Gluconic	Oxidation C_1	0	L-Arabinose	Inversion C_4	0
D-Galactose	Inversion C_4	0			
D-Mannose	Inversion C_2	6,700			

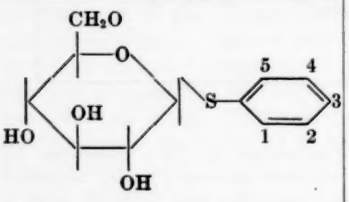
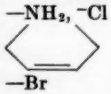
* Modification with respect to the D-glucose configuration.

† Conditions for the K_i determination were the same as in Fig. 3.

‡ Modification with respect to the D-xylose configurations.

TABLE III

Affinities of β-D-thioglucosides for β-glucosidase

Substitution			K_i* × 10^5
	Position	Group	
			456
	4	—OCH_3	190
	4	—CH_3	130
	4	—OCH_2CH_3	72
	4	—$N(CH_2CH_3)_2$	69
	2, 4	—NH_2, —Cl	66
	1, 2		35.6
	4	—Br	35.0
	2, 5	—Cl, —Cl	22.2
	3, 4	—Cl, —CH_3	13.5
Phenyl-thio-β-D-galactoside			9000

* Conditions for the K_i determination were the same as in Fig. 3.

(21) yielded a straight line (Fig. 3). The K_s value was calculated to be 8.05×10^{-5} M.

Enzyme Specificity

The enzyme specificity of yeast β-glucosidase was examined by measuring the capacity of the enzyme to form complexes with a series of related substrates and to hydrolyze a series of glycosides. The affinities of various glycosides for β-glucosidase were measured in a series of tests in which the inhibition of NPG hydrolysis by a fixed concentration of inhibitor was tested at various NPG concentrations. From the initial velocity (v_i) of NPG hydrolysis (Fig. 3), a K_i value (competitive inhibition) was calculated for various substances (I) from the following equation:

$$\frac{1}{v_i} = \frac{1}{v_m}\left(K_s + \frac{K_s I}{K_i}\right)\frac{1}{S} + \frac{1}{V_m}$$

where K_s is the Michaelis-Menten constant and V_m is the maximal rate of hydrolysis for NPG. The K_i values for the enzyme of various hexoses and pentoses are given in Table II, of thio-β-glucosides in Table III and of alkyl- and aryl-β-glucosides in Tables IV and V. Included in Tables IV and V are direct estimations of the maximal velocity (V_m) of hydrolysis of the various glucosides.

The complex-forming capacity of phenyl-α-D-glucoside and phenyl-thio-β-D-galactoside could not be attributed to contaminating glucose. The failure of the enzyme to hydrolyze phenyl-α-D-glucoside or *p*-nitrophenyl-α-D-glucoside indicates the absence of α-glucosidase activity from the enzyme preparation.

Effect of pH and Other Factors

pH—The rate of NPG hydrolysis was studied as a function of pH with potassium phosphate-succinate buffer. The enzyme had a wide pH range for activity with an optimal pH range of 6.4 to 6.8 (Fig. 4).

With the use of the NPG assay, the relation between K_m and pH has been determined over the pH range of 6.0 to 8.9. Contrary to the biphasic curve obtained for yeast α-glucosidase (19), the pK_m was essentially independent of pH over the range studied.

The effect of pH on the stability of the enzyme was examined by incubation for 2 hours at 30° at various pH values in the absence of substrate. The enzyme was stable from pH 6 to 8.0. On the other hand, preincubation at pH 5.5 and 5.1 resulted in 20 per cent and 31 per cent loss, respectively.

Temperature—The effect of temperature on the rate of hydrolysis was examined over the range of 5–65°. The results, plotted in the conventional Arrhenius manner (log k versus $1/T$; k = zero order velocity constant, T = absolute temperature), are shown in Fig. 5.

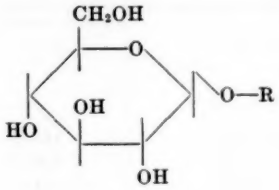
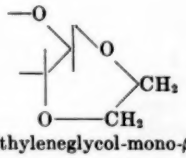
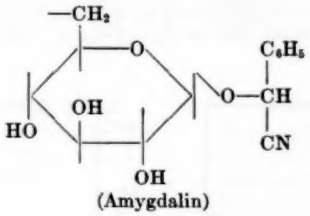
A deviation from linearity is observed only above 45°. From these data the average energy of activation (ΔH^*) over the range of 10–40° was 16,600 cal. per mole.

Ion Effects—Purified β-glucosidase (Fraction VI) was dialyzed for 8 hours against 0.001 M Versene (disodium salt of ethylendiaminetetraacetic acid, Dow Chemical Company), pH 6.8, and for 24 hours against 10^{-4} M mercaptoethanol, after which it was diluted 1000-fold into 0.05 M Tris-succinate buffer, pH 6.8. The rate of NPG hydrolysis was unaffected by the addition of K^+, Na^+, NH_4^+ or Li^+ chlorides up to concentrations of 0.1 M.

Among the divalent cations (Table VI), Ca^{++}, Mg^{++}, and Mn^+ are without appreciable effect on the activity of the enzyme at 0.01 M. An examination of the heavy metals (0.01 M) indicated that β-glucosidase was sensitive to Fe^{+++}, Hg^{++}, Pb^{++}, Zn^{++}, Cu^{++}, and Co^{++}. Similar results have been reported for

TABLE IV

*Maximal rates of hydrolysis of alkyl-β-D-glucosides and their affinities for β-glucosidase**

R	K_i† × 10^5	V_m‡
		μmole/mg. protein/10 min.
	15,000	12.1
—CH_3	5,600	33.2
—$CH_2CH{=}CH_2$	4,500	47.5
 (Ethyleneglycol-mono-β-)	1,600	
 (Amygdalin)	1,410	0
—$(CH_2)_9CH_3$	6.3	217.0

* The following glucosides are neither complex-forming compounds nor substrates: diethyl-mono-β-D-glucoside, methyl-β-D-xyloside, methyl-α-D-glucoside, ethyl-α-D-glucoside, butyl-α-D-glucoside, and sucrose.

† Conditions for the K_i determination are the same as in Fig. 3.

‡ V_m values were determined by either of the two methods as indicated in the text: (*a*) by a spectrophotometric method in which 370 units of β-glucosidase of Fraction VI were used per 1 ml. of reaction mixture, or (*b*) manometrically by a *Torula monosa* fermentation in which 665 units of β-glucosidase of Fraction VI were used per 2 ml. of reaction mixture.

β-glucosidase of almond (4), α-glucosidase of yeast (19) and invertase of yeast (22).

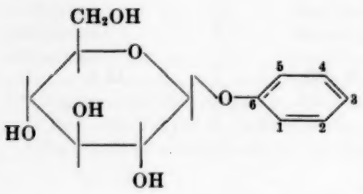
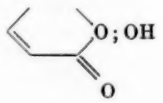
Sulfhydryl Inhibitors—The instability of the enzyme in the presence of heavy metals led us to examine the effect of sulfhydryl inhibitors on enzyme activity. As shown, CMB at a concentration of 1×10^{-4} M (Table VII) almost completely inhibits enzyme activity. Iodoacetate is a less effective inhibitor. Cysteine reversed the inhibition by both CMB and iodoacetate.

Although observations of the above variety show the presence of —SH groups on the enzyme, they do not indicate whether one or more of these —SH groups participates in the combination between substrate and enzyme. If the latter were true one would expect a competition between substrate and sulfhydryl agents for the —SH group involved in the catalytic reaction. Such competition should reflect the activity of the substrate with respect to complex formation with the enzyme. This was tested by examining the protecting effects of various substrates on the rate of inactivation of β-glucosidase by CMB. The results are shown in Fig. 6. In the presence of CMB a first order rate of inactivation (−0.064) of the single hit variety occurs indicating the presence of an essential —SH site. The decreasing first order rates of inactivation in the presence of cellobiose (−0.054), phenyl-β-D-glucoside (−0.020) and NPG (−0.012) progressively follow their respective dissociation constants: 1.5×10^{-1} M, 1.58×10^{-3} M and 8.0×10^{-5} M.

The competitive nature of the protecting effect of substrate against CMB inactivation is further indicated by the concentration relationship between substrate and CMB (Table VIII). These results show that increasing the substrate concentration decreases the rate of inactivation by CMB. Furthermore, the rate of reaction of CMB with the —SH group involved in enzyme catalysis is generally determined by the ratio of substrate to CMB rather than the concentration of CMB itself. For example, the rate of inactivation of enzyme by phenyl-β-glucoside to the CMB ratio of 4.5×10^2 (5×10^{-3} M to 1.25×10^{-5} M;

TABLE V

*Maximal rates of hydrolysis of aryl-β-D-glucosides and their affinities for β-glucosidase**

Substitution			K_i† × 10^5	V_m†
	Position	Group		μmole/mg. protein/10 m.ni
	0	O	158	163.5
	6	—CH_2—	380	116.0
	(Salicin) 5	—CH_2OH	264	81.8
	(Arbutin) 3	—OH	173	187.0
	1, 2, 3, 4, 5, and 6	—H	68	145.0
	3	—$(CH_2)_2CH_3$	8.8	196.0
	(NPG) 3	—NO_2	8.0‡	126.0
	4	—C_6H_5	3.6	
	(Esculin) 1, 2; 5			63.6
Phenyl-α-D-glucoside	0	0	160	0

* The following compounds are not hydrolyzed: *p*-nitrophenyl-α-D-glucoside and *o*-nitrophenyl-β-D-galactoside.
† Conditions for the determination of K_i and V_m are the same as those given in Table IV.
‡ K_s.

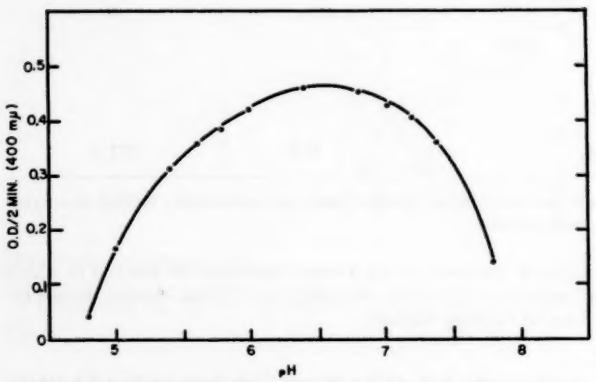


FIG. 4. NPG hydrolysis as a function of pH. The reaction mixture (1.0 ml.) contained 1×10^{-3} M NPG, 0.067 M potassium phosphate-succinate buffer at the desired pH, and 225 units of β-glucosidase (Fraction VI). The assay was carried out by the discontinuous method, as indicated in Fig. 3. *O.D.* = optical density.

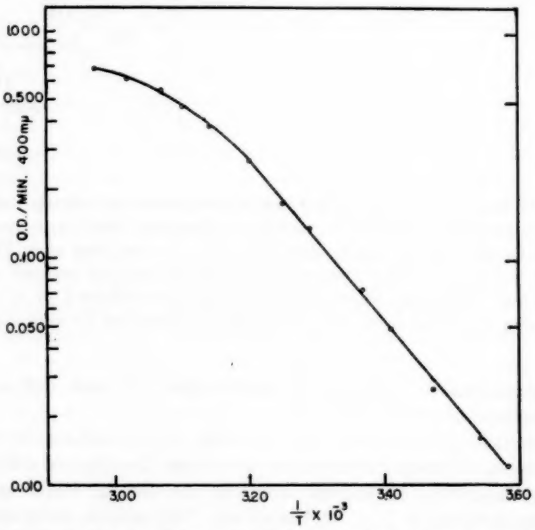


FIG. 5. Arrhenius plot of NPG hydrolysis. The standard assay mixture, as indicated in the text, was used with the addition of 135 units of β-glucosidase from Fraction VI. The temperature was varied as indicated. *T* is the absolute temperature. *O.D.* = optical density.

10^{-2} M to 1.87×10^{-5} M) is −0.027 to −0.0298, whereas at CMB concentrations of 1.25×10^{-5} M or 1.87×10^{-5} M the inactivation rates are −0.0418 and −0.0718, respectively.

DISCUSSION

Several types of enzymes active on β-glucosides in microorganisms have been described. *Clostridium thermocellum* contains a cellobiose phosphorylase (23) which reversibly catalyzes the conversion of cellobiose to glucose and glucose-1-phosphate. Takano and Miwa (24) have demonstrated a glucotransferase activity for purified β-glucosidase of *Aspergillus niger* and *Penicillium chrysogenum*. Fungi, however, possesses a β-glucosidase active against β-thioglucosides (5, 25). Although the purified β-glucosidase of *S. cerevisiae* strain Yeast Foam catalyzes the hydrolysis of a number of alkyl- and aryl-β-D-glucoside, β-thioglucosides function as agents for complex formation with the enzyme but not as substrates. The reaction

TABLE VI

Inhibition of β-glucosidase by various cations

The reaction mixture (3.0 ml.) contained 0.05 M Tris-succinate buffer, pH 6.8, 150 units of β-glucosidase of Fraction VI, cation as indicated, and 1×10^{-3} M NPG. The NPG was added after a 20-minute preincubation period of other components at 30°.

Cation	Concentration	Inhibition
	M	%
None		0
Ca^{++}	1×10^{-3}	5
Mg^{++}	1×10^{-3}	6
Mn^{++}	1×10^{-3}	5
Cu^{++}	3.33×10^{-5}	60
Fe^{+++}	3.33×10^{-4}	52
Zn^{++}	3.33×10^{-4}	17
Co^{++}	3.33×10^{-4}	17
Ag^{+}	3.33×10^{-5}	100
Hg^{+}	3.33×10^{-4}	66
Pb^{++}	3.33×10^{-6}	20

TABLE VII

Effects of sulfhydryl agents on β-glucosidase

Reaction mixture (3.0 ml.) contained 0.067 M phosphate buffer, pH 6.8, 150 units of β-glucosidase of Fraction VI, and inhibitor as indicated. NPG at 1×10^{-3} M and cysteine were added after a 10-minute preincubation period of β-glucosidase with the inhibitors at 30°.

Inhibitor	Concentration	Cysteine	Inhibition
	M	mg.	%
None		0	0
		1	0
CMB	3.35×10^{-5}	0	59
	3.35×10^{-5}	1	18
	1×10^{-4}	0	95
	1×10^{-4}	1	71
Iodoacetate	1×10^{-4}	0	29
	1×10^{-4}	1	8

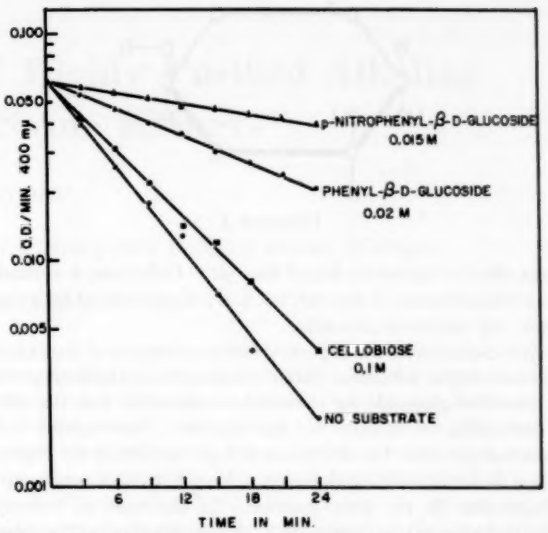


FIG. 6. Effects of substrates on the inactivation of β-glucosidase by CMB. 2 ml. of reaction mixture contained 0.067 M phosphate buffer, pH 6.8, and 900 units of β-glucosidase (Fraction VI), 1×10^{-5} M CMB. The addition of substrates is as indicated. At intervals 0.1 ml. was removed and immediately assayed in the absence of glutathione. *O.D.* = optical density.

TABLE VIII

Substrate protection against sulfhydryl inactivation

The reaction mixture (1.0 ml.) contained 0.067 M phosphate buffer, pH 6.8, CMB and phenyl-β-D-glucoside at the indicated concentrations, and 1150 units of β-glucosidase (Fraction VI). The incubation temperature was 30°. At intervals 0.1 ml. was added to the assay mixture minus glutathione, and this mixture was assayed as described in the text.

	Phenyl-β-D-glucoside		
CMB	Zero M	5×10^{-3} M	1×10^{-3} M
M	k^*	k^*	k^*
9.4×10^{-6}	−0.0242	−0.0170	−0.0140
1.25×10^{-5}	−0.0418	−0.0270	−0.0172
1.56×10^{-5}	−0.0616	−0.0321	−0.0230
1.87×10^{-5}	−0.0718	−0.0370	−0.0298

* k is the rate of inactivation.

is strictly hydrolytic and therefore more analogous to β-glucosidase of almond (4).

Yeast β-glucosidase displays a high degree of specificity to sugars. The specificity with regard to carbon atom 1 appears to be absolute. Oxidation of C_1 to —COOH (gluconic acid) abolishes the affinity for the enzyme. α-Glucosides are neither substrates nor complex-forming agents for the enzyme, with the exception of phenyl-α-D-glucoside. The steric configuration about C_2 of the D-glucopyranoside ring is not important in determining enzyme affinity. D-Glucose and D-mannose (inversion at C_2) are both competitive inhibitors with K_i values of 8.5×10^{-3} M and 6.7×10^{-2} M, respectively. Similar observations have been reported for β-glucosidase of *Stachybotrys atra* (5). The hydrolysis of β-mannosides by the β-glucosidase of yeast has not as yet been examined. Studies with other β-glucosidase systems (4, 5) have reported that although a hydroxyl group must be attached to C_4 for affinity, its configuration about C_4 is immaterial. In the present experiments, absolute specificity at C_4 was observed since β-galactosides are not substrates of the enzyme and galactose itself does not compete with the hydrolysis of NPG. The specificity with regard to C_6 is not absolute since either oxidation of C_6 to —COOH (glucuronic acid) or replacement of $-CH_2OH$ by H (D-xylose) only increases the dissociation constant for the enzyme ($K_i = 1.05 \times 10^{-1}$ M). Based on the behavior of D-xylose, the configuration about C_3 must be that of D-glucose since inversion of the hydroxyl group at C_3 in a C_5 sugar (D-ribose) eliminates affinity for the enzyme. From these observations, the essential configuration required for a molecule to form complexes with β-glucosidase of yeast is given by the portion of the D-glucose molecule as shown in Diagram 1. A high degree of tolerance to substitution in the aglycon has been characteristic of various β-glucosides (4, 26). β-Glucosidase of yeast is active against both alkyl- and aryl-β-glucosides. Among the β-alkyl series, the yeast enzyme displays a progressively increasing rate of hydrolysis and affinity with the increasing chain length of the aglycon group. *n*-Decyl-β-glucoside is the

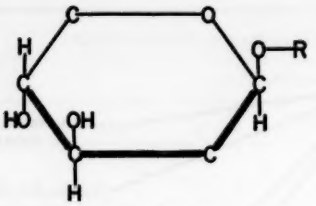


DIAGRAM 1

most effective substrate found thus far. Cellobiose, a naturally occurring substrate of the enzyme, had a slower rate of hydrolysis than did methyl-β-glucoside.

Aryl-β-glucosides are in general better substrates of the enzyme and have higher affinities. Meta substitution in the benzene ring of phenyl-β-glucoside or phenyl-β-thioglucoside has the effect of increasing the affinity for the enzyme. For example, 4-biphenyl-β-glucoside has 200 times as high an affinity for β-glucosidase as does phenyl-β-glucoside. A similar effect occurs upon substitution in the para position. In the case of 3-propylphenyl-β-glucoside an increased V_m is also observed. The substitution of a chlorine atom at the para position of 4-methylphenyl-β-thioglucoside decreases the K_i from 1.3×10^{-3} M to 1.35×10^{-4} M. Reduction of the benzene ring increases the affinity for the enzyme without significant effect upon its rate of hydrolysis. Substitution of the oxygen of the glycoside link by sulfur abolishes sensitivity to hydrolysis and decreases affinity for the enzyme.

Although various mechanisms have been proposed for the hydrolysis of β-glucosides (26) there is little information available on the nature of the groups involved. The inactivation of various β-glucosidases by salts of heavy metals (4) and the precipitation of the enzyme by silver ions (27) has indicated the presence of —SH groups on β-glucosidases. The inactivation of yeast β-glucosidase by heavy metals which are sulfhydryl binding agents, and its reversal by cysteine, indicates that similar —SH groups are present. The possible involvement of some of these —SH groups in the catalysis of substrate hydrolysis is indicated by the competitive substrate protection against inactivation by CMB. The degree of protection afforded is dependent upon the affinity of the substrate for the enzyme. Attempts to detect ionizable groups on the enzyme involved in catalysis over the pH range of 6 to 8.9 have been negative, and thus presumably the ionizing group (pK_a 6.6 to 6.8) which is present in α-glucosidase of yeast (19) is absent in β-glucosidase.

SUMMARY

An inducible enzyme that catalyzes the hydrolysis of aryl- and alkyl-β-D-glucosides has been purified 120-fold from extracts of *Saccharomyces cerevisiae* strain Yeast Foam. β-Thio-D-glucosides function as agents for complex formation with the enzyme but not as substrates. An absolute specificity at carbon atoms 1, 3, and 4 of β-D-glucosides is required for enzyme affinity. One or more sulfhydryl groups have been identified as being involved in the over-all catalysis of substrate hydrolysis.

Acknowledgments—We are indebted to Dr. Robert Bock for determining the sedimentation constant of the enzyme and to Mr. Ray Epstein for assistance with certain experiments.

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Preparation and Properties of Highly Purified Alkaline Phosphatase from Swine Kidneys*

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(Received for publication, June 23, 1958)

This report concludes an investigation directed toward the isolation of alkaline phosphatase (1, 2). Highly purified preparations were desired for the unequivocal characterization of this enzyme. The degree of purification obtained is indicated by the fact that the specific activity of the enzyme in the source tissue is approximately 1 unit per mg. of total nitrogen, whereas that of our most active preparations was 10,500 units per mg. of protein nitrogen. Furthermore, our better material appeared to contain 80 to 90 per cent of one component as judged by analysis in the ultracentrifuge and by free electrophoresis. Evidence is also presented for a close association between the activity of the enzyme and its content of zinc. Fully active preparations of maximal purity contained 0.15 to 0.18 per cent of zinc.

EXPERIMENTAL

Enzyme Assay—Activity was estimated by the procedure of King *et al.* (3) with only minor modification (1, 4). All assays were performed in the presence of magnesium for activation unless otherwise indicated. The routine assay also included preliminary activation by incubation of the final enzyme dilution for 3 hours at 25° in 0.01 M DL-alanine buffered at pH 9.7 with 0.05 M carbonate. The enzyme unit is defined as that amount of enzyme which will liberate 1 mg. of phenol from disodium phenyl phosphate in 15 minutes at 25°. The pH optimum and kinetics of substrate hydrolysis were redetermined, and it was found that the assay, as developed with crude enzyme preparations, was a valid measure of activity for highly purified, amino acid-activated enzyme.

Protein—Protein nitrogen was determined as indicated previously (1) for the data in Procedure I (see below) and by the procedure of Lowry *et al.* (5) for the remaining data. The reproducibility of the latter determination was satisfactory when the various reaction steps were carried out with careful timing at 25°. Twice crystallized ovalbumin was used as a standard protein, and occasional Kjeldahl determinations (6) were carried out on enzyme solutions to assure the validity of the use of ovalbumin as a reference protein. The stock ovalbumin solution was adequately stable when prepared in 50 per cent aqueous glycerol and stored at −20°.

Activation Studies—The procedure for testing the activation efficiency of the various compounds was as follows. Stock enzyme solution[1] was diluted to approximately 0.2 units per ml. in the solution to be tested.[2] After the solution was incubated at 25° for the desired length of time, its activity was determined. Except where otherwise stated, the incubation time was 3 hours, the activator was 0.01 M, and the buffer was 0.05 M carbonate adjusted to pH 9.7. For the control incubations the activator was omitted. The chromogenicity of each of the compounds in the assay was determined as were also the possible effects of these compounds on the chromogenicity of phenol. The amino acids and other compounds tested were in the purest state obtainable and, when necessary, were recrystallized.

Zinc—For quantitative zinc determinations, enzyme samples were digested with a small amount of glass-redistilled concentrated sulfuric acid in quartz tubes. When the digest became clear, the remaining acid was neutralized by exposing it to ammonia gas, and the solution was evaporated to dryness, dissolved in 0.01 N HCl, and analyzed by the dithizone procedure (7).

RESULTS

Isolation of Enzyme

Preparation of a crude concentrate of the enzyme is outlined in Procedure I. The basic steps employed are solubilization of the enzyme with pancreatin, thermal denaturation of contaminating proteins at room temperature in the presence of 24 per cent ethanol, fractional precipitation with ethanol at 5°, fractional precipitation with ammonium sulfate at 5°, and dialysis.

The dialyzed enzyme solution is then carefully titrated to pH 4.75 at 0–5° with acetic acid, and the precipitate, containing the enzyme, is separated by high speed centrifugation (Procedure II). Alkaline phosphatase is relatively unstable at acid pH values, therefore this step is carried out at low temperature and the precipitate is promptly dissolved in 0.01 M $NaHCO_3$. Occasionally, preparations were obtained that gave little precipitation at this

* A summary of this investigation was reported at the meeting of the American Society of Biological Chemists at Atlantic City in April, 1954 (*Federation Proc.*, **13**, 260 (1954)).

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[1] The stock solutions used were prepared to require dilutions of 1:500 or 1:1000 in order to yield activities suitable for assay. The stock solution solvent, which greatly stabilized the enzyme during storage at 5°, was 0.05 ionic strength phosphate buffer of pH 7.2, prepared in 50 per cent aqueous glycerol.

[2] The manner of cleaning the glassware and the type of glass used are factors to be considered. Pyrex tubes, cleaned with commercial detergent (Alconox), gave greater control activation than Pyrex tubes cleaned with chromic acid cleaning solution or nitric-sulfuric acid cleaning solution, even when the latter was prepared with glass-redistilled acids. Deliberate extensive etching of acid-cleaned Pyrex tubes greatly reduced control activations.

stage. This was overcome by the use of a different lot of pancreatin, preferably one which had been aged for a few weeks. Attempts to carry out this step with the undialyzed extract resulted in no precipitation, indicating that the presence of ammonium sulfate interfered, presumably by means of a nonspecific salt effect. The precipitation is probably dependent upon the formation of an insoluble complex with some other component in the mixture, possibly nucleic acid, since Albers' preparations (8) of horse kidney alkaline phosphatase gave very little precipitation when treated in this manner. Repeated acid precipitation did not increase the purity of the enzyme.

The third stage in the purification of the enzyme is outlined in Procedure III. Inactive protein along with most of the brown pigmentation was removed by adsorption on $Ca_3(PO_4)_2$ gel (9). This step must be carried out with care since adsorption of the enzyme on the gel can result in the loss of the entire preparation. The resulting solution is treated with kaolin to remove sufficient proteolytic activity to allow protamine precipitation. Excess protamine is then removed by autolysis followed by adsorption on kaolin. The final solution is concentrated by ammonium sulfate precipitation.

Additional $(NH_4)_2SO_4$ fractionation of the concentrate described in Procedure I was not very effective. However, the treatments outlined in Procedures II and III gave a preparation that could be fractionated with saturated $(NH_4)_2SO_4$, an example of which is described in Procedure IV. Occasionally two consecutive fractionations were required to obtain material with the specific activity obtained in Step 5. Less active preparations were stored and added to subsequent purifications at an appropriate stage of specific activity or were simply refractionated. Continued application of this scheme of $(NH_4)_2SO_4$ fractionation has yielded preparations with measurements of specific activity up to 5000.

Procedure V outlines the preparation of material with a specific activity of 8000 starting from 1800. This purification was obtained with a combination of Sevag's $CHCl_3$ treatment (10) with electrophoresis-convection (11). The enzyme is relatively resistant to surface denaturation and thus allows removal of a significant proportion of the more sensitive contaminating protein by emulsification with $CHCl_3$. An example of the final steps in the preparation of enzyme of maximal activity is presented in Procedure VI. Generally, the specific activity was increased from 1000 to between 2000 and 3000 by ammonium sulfate fractionation, from between 2000 and 3000 to between 4000 and 7000 by treatment with $CHCl_3$, and final purification was obtained with electrophoresis-convection.

Procedures—In the descriptions which follow PU is phosphatase units, TPKU is the total phosphatase units $\times 10^{-3}$, and PU/mg. PN is units per mg. of protein nitrogen.

Procedure I—Preparation of Crude Concentrate of Alkaline Phosphatase[3]

	TPKU	PU/mg. PN
1. Mix 8 kg. of ground swine kidney cortex, 4 l. of H_2O, 400 ml. of toluene, 160 gm. of Viokase,[4] and 200 ml. of 20 per cent Na_2CO_3	242	2.69[5]
2. Digest overnight at room temperature, and then add another 200 ml. of 20 per cent Na_2CO_3. Digest for 2 more days	192	4.14
3. Add 4 l. of 95 per cent ethanol, shake at room temperature for 4 hours, and then let stand overnight	202	5.12
4. Add 1.6 kg. of Hyflo Super-Cel, and filter with suction (2 l. of suspension per filtration with 32-cm.-diameter Buchner funnels). The filter cakes are rinsed with a total of 2 l. of 24 per cent ethanol. Filtrate	142	15.1
5. Add 25 ml. of cold 95 per cent ethanol per 100 ml. of filtrate at 5° slowly with stirring. Add 200 gm. of Hyflo Super-Cel and filter with suction. Wash the cake with 800 ml. of 38 per cent ethanol. Filtrate	142	20.0
6. Slowly add 60 ml. of cold 95 per cent ethanol per 125 ml. of filtrate as above. Add 160 gm. of Hyflo Super-Cel and filter with suction. Wash the filter cake with 800 ml. of cold 57 per cent ethanol, and discard the filtrate and washings. Stir the filter cake with 3.2 l. of 0.1 M $NaHCO_3$ for 30 minutes at 5° and filter with suction. Wash the filter cake twice with 400 ml. of H_2O. Filtrate	117	62.2
7. Add 25 gm. of solid $(NH_4)_2SO_4$ per 100 ml. of filtrate, and dissolve with slow stirring at 5°. Add 80 gm. of Hyflo Super-Cel and filter with suction. Wash the filter cake with 800 ml. of H_2O in which 192 gm. of $(NH_4)_2SO_4$ has been dissolved. Filtrate	128	121
8. Add 30 gm. of $(NH_4)_2SO_4$ per 112.5 ml. of filtrate and dissolve as above. Add 30 gm. of Hyflo Super-Cel and filter with suction. Wash the filter cake with 400 ml. of H_2O in which 224 gm. of $(NH_4)_2SO_4$ has been dissolved. Discard the filtrate. Stir the filter cake with 400 ml. of 0.05 M $NaHCO_3$ for 30 minutes at 5°. Filter with suction, and wash the filter cake with H_2O to give 500 ml. of filtrate plus washings. Dialyze the filtrate against 0.1 per cent and finally 0.05 per cent $NaHCO_3$ at 5° until free of sulfate	106	173

Procedure II—Acid Precipitation of Alkaline Phosphatase Crude Concentrate

	PU/ml.	TPKU	PU/mg. PN
1. Dialyzed alkaline phosphatase crude concentrate	179	135	180
2. Enzyme solution cooled to 0° and titrated with efficient stirring to pH 4.75 with M acetic acid. The suspension was then centrifuged at 60,000 $\times$ *g* for 20 minutes at 5°. The supernatant was discarded, and the precipitate was taken up in 25 ml. of 0.1 M $NaHCO_3$. The solution was rinsed from the centrifuge bowl with 0.001 M $NaHCO_3$ to give a final total volume of 50 ml.	2300	115	448

[3] The values noted in this procedure were obtained without alanine activation.
[4] Viobin Laboratories, 4 × U.S.P. pancreatin.
[5] 1.19 units per mg. of total nitrogen.

Procedure III—Preparation of Purified Concentrate of Alkaline Phosphatase

	PU/ml.	TPKU	PU/mg. PN
1. The acid-precipitated crude concentrate from 16 kg. of cortex is cooled to 5°.	1200	197	478
2. Stir for 30 minutes with 30 ml. of 8 to 10 per cent $Ca_3(PO_4)_2$ gel. The gel is then removed and washed twice with 0.001 M $NaHCO_3$ by centrifugation at 5°. Supernatant	922	184	623
3. The solution is titrated to pH 8 with 20 per cent Na_2CO_3, cooled to 5°, and treated with 10 ml. of gel as above. Supernatant	724	171	662
4. The supernatant is stirred for 30 min. at 5° with 20 gm. of acid-washed kaolin. The kaolin is removed and washed by centrifugation.[6] A 2 per cent dialyzed protamine sulfate solution is added to the enzyme solution at room temperature to the point at which there is no additional precipitation. The suspension is immediately centrifuged for 5 minutes, and the clear supernatant is allowed to stand at room temperature for 1 hour. After repeating the above kaolin treatment, 65 gm. of $(NH_4)_2SO_4$ are added per 100 ml. of enzyme solution and dissolved slowly at 5°. The precipitated enzyme is removed by suction filtration with the aid of 1 gm. of Hyflo Super-Cel. The filter cake is extracted with 20 ml. of 0.05 M $NaHCO_3$ for 30 minutes, and the resulting extract is dialyzed overnight against 0.1 per cent $NaHCO_3$. Extract	2580	144	1008

Procedure IV—Fractionation of Alkaline Phosphatase with Saturated Ammonium Sulfate at 5°

	PU/ml.	TPKU	PU/mg. PN
1. The dialyzed enzyme solutions from several preparations were combined	5,300	403	903
2. Brought to 0.55 saturation[7] with solid $(NH_4)_2SO_4$. 1 gm. of Hyflo Super-Cel was added and the preparation was filtered with suction.[8] Precipitate extract[9]	697	9.4	214
3. The 0.55 saturated filtrate was taken to 0.60 saturation with saturated $(NH_4)_2SO_4$.[10] 0.5 gm. of Hyflo Super-Cel was added and the preparation was filtered with suction. Precipitate extract	2,490	22.4	535
4. The 0.60 saturated filtrate was taken to 0.65 saturation as in Step 3. Precipitate extract	4,850	53.4	1,020
5. The 0.65 saturated filtrate was taken to 0.70 saturation as in Step 3. Precipitate extract	17,950	196	2,550
6. The 0.70 saturated filtrate was taken to 0.80 saturation with solid $(NH_4)_2SO_4$ as in Step 2. Precipitate extract	8,490	114	606

Procedure V—Purification by Electrophoresis-Convection and Agitation with Chloroform

	PU/ml.	TPKU	PU/mg. PN
1. A number of preparations were pooled and dialyzed against 0.01 M alanine in 0.1 ionic strength phosphate buffer of pH 7.2. Enzyme solution	2900	860	1880
2. Agitated vigorously with an equal volume of $CHCl_3$ for 30 minutes at room temperature. Concentrated the enzyme solution and rinsings by ultrafiltration	3010	800	2740
3. Repeated Step 2. Concentrated enzyme solution	4600	792	4110
4. Fractionated three times by electrophoresis-convection, stripping out three top-cuts.[11] Final bottom-cut	3340	340	3350
5. Treated bottom-cut as in Step 2	2340	290	3880
6. The $CHCl_3$ treated bottom-cut was then fractionated by electrophoresis-convection to obtain two more top-cuts. Final bottom-cut	2230	210	3500
7. The five top-cuts from Steps 4 and 6 were combined, concentrated by ultrafiltration, and refractionated by electrophoresis-convection. The bottom-cut was treated with $CHCl_3$ as in Step 2 and then refractionated by electrophoresis-convection. Final bottom-cut	3380	253	5620
8. The two top-cuts from Step 7 were combined and concentrated by ultrafiltration	12,060	148	8290

[6] Test for trypsin by adding 0.5 ml. of the protamine solution to 5 ml. of the supernatant. If the precipitate redissolves significantly within 10 minutes, the kaolin treatment must be repeated.

[7] Expressed in terms of saturation at 0°, based on a solubility of 70.6 gm. per 100 ml. of water.

[8] The filter cakes and glassware in this and subsequent precipitation steps were rinsed with a small volume of saturated $(NH_4)_2SO_4$ solution of the appropriate concentration.

[9] Precipitate extracts were prepared by stirring the filter cake with a small volume of 0.1 ionic strength phosphate buffer of pH 7.2 for 30 minutes, filtering with suction, and rinsing with the same buffer.

[10] The saturated $(NH_4)_2SO_4$ solution was added very slowly with constant stirring over a period of several hours. This was accomplished by the use of a syringe equipped with a mechanical drive. In this manner volumes as small as 2 ml. could be added over a period of 4 to 5 hours.

[11] The electrophoretic fractionations were carried out with the use of 0.1 ionic strength phosphate buffer of pH 7.2 and a field strength of 1.6 volts per cm., for 16 to 20 hours at 5°. Repeated fractionation involved removal of the top-cut at the end of a run, dilution of the remaining solution to fill the cell, and repetition of the electrophoresis.

Procedure VI—Preparation of Enzyme with Maximal Specific Activity

	PU/ml.	TPKU	PU/mg. PN
1. An enzyme preparation was dialyzed against 0.01 M alanine in 0.1 ionic strength phosphate buffer of pH 7.2	1915	322	5820
2. Shook vigorously for 15 minutes with an equal volume of $CHCl_3$ at room temperature. Aqueous layer plus rinsings	1120	289	7340
3. Concentrated by ultrafiltration and fractionated by electrophoresis-convection at 5° and 1.6 volts per cm. for 20 hours. Bottom-cut	2535	225	8370
Top-cut	995	60	10,500

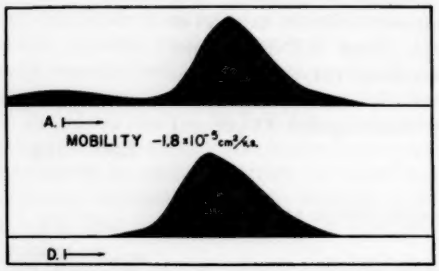

FIG. 1. Electrophoretic pattern of enzyme with an activity of 9430 phosphatase units per mg. of protein nitrogen. Conditions of electrophoresis involved 1.6 mg. of protein nitrogen per ml., 0.1 ionic strength phosphate buffer of pH 7.2, a temperature of 5°, 2.5 hours of migration.

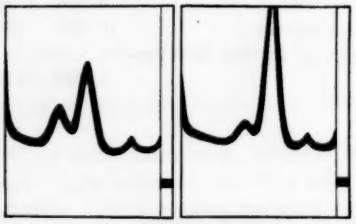

FIG. 2. Ultracentrifuge patterns of enzyme preparations with activities of 5620 and 8290 phosphatase units per mg. of protein nitrogen, respectively. Conditions of ultracentrifugation involved 1.2 and 1.5 mg. of protein nitrogen per ml., respectively; solvent, 0.01 M DL-alanine in 0.1 ionic strength phosphate buffer of pH 7.2; 70 minutes at 30°; 52,640 r.p.m. rotor speed.

Physicochemical Characterization

4 ml. of the preparation from Step 8 of Procedure V were dialyzed for 24 hours against glass-redistilled water adjusted to pH 7.25 with 0.2 M Na_2CO_3, lyophilized in a quartz tube, and analyzed for elementary composition.[12] The percentages obtained were 45.03, 7.21, 12.61, 1.21, and 1.69 for carbon, hydrogen, nitrogen, sulfur, and ash, respectively.

Electrophoretic analysis[13] of a preparation with a specific activity of 9430 units per mg. of protein nitrogen is presented in Fig. 1. At pH 7.2 the isolated protein migrated as a single component. However, analysis in the ultracentrifuge[14] readily separated this type of preparation into three distinct components (Fig. 2). The specific activities of the two preparations analyzed in the ultracentrifuge were 5620 and 8290 (Procedure V). The increase in the major component is directly proportional to the increase in specific activity. The specific activity of the pure enzyme would be calculated to be in the range of 10,000 to 11,000, if one assumed that the major component represents pure material. This range is in agreement with the activities of our best preparations.

Amino Acid Activation

A systematic study of the amino acid activation of our highly purified preparations was carried out. Over 50 compounds, including a wide variety of amino acids, peptides, their derivatives, and analogous compounds were employed. Results obtained with some of the compounds tested are given in Table I. Considering first the effect of increasing separation of the amino and carboxyl groups, one sees that the α-configuration was by far the most effective. The β-configuration gave intermediate activation whereas further separation (γ-aminobutyric to ϵ-aminocaproic acids) resulted in complete loss of capacity for activation. The requirement for α-amino group hydrogen atoms was considered next, and it was observed that substitution by one or two methyl groups was possible without reduced activation (sarcosine and dimethylglycine). Further methylation (betaine) resulted in a completely inactive structure.

Activation by α-aminoisobutyric acid indicates that replacement of the hydrogen at the α-carbon does not impair the power to activate, whereas activation by α-amino-*n*-valeric acid, α-amino-*n*-butyric acid, α-alanine, and norleucine demonstrates the lack of dependence upon the length of the hydrocarbon chain. Acyl substitution on the amino group has a marked effect, as can be seen by the lack of activation by hippuric acid, acetylglycine, benzoylglycylglycine, and acetylglycylglycine. Glycine anhydride seemed to be a moderately effective activator; however, its effect decreased with decreasing pH which indicated that hydrolysis to glycylglycine, an efficient activator, may have taken place in the more alkaline test solutions.

Glycine ethyl ester, taurine, 3-aminopropanol, glucosamine, imidazole, and histamine are of particular interest for testing the effects of carboxyl group modification or substitution. The activation effect of these compounds indicates that sulfonic acid, carbonyl, hydroxyl, and unsubstituted heterocyclic nitrogen groups may substitute for the carboxyl in the activating molecule.

Results with aminopropanol, taurine, and β-alanine indicated that β-ethanolamine and aminomethanesulfonic acid might activate. These two compounds were then found to be moderately effective. 2-Amino-2-methyl-1,3-propanediol and 2-amino-2-(hydroxymethyl)1,3-propanediol, analogues of β-ethanolamine

[12] Analyses were performed by Micro-Tech Laboratories, Skokie, Illinois.

[13] The electrophoretic analysis was carried out by J. M. Vandenbelt and R. B. Scott of Parke, Davis and Company. Their invaluable assistance is gratefully acknowledged.

[14] Obtained through the generous assistance of V. Schelling and D. H. Basinski of the Department of Laboratories of the Henry Ford Hospital.

TABLE I

*Activation of alkaline phosphatase by preliminary incubation with various compounds**

Compound	Activity	Compound	Activity	Compound	Activity
N-acetylglucosamine	46	α-Amino-*n*-valeric acid	165	Glycylglycylglycine	144
Acetylglycine	35	D-Aspartic acid	108	Hippuric acid	37
Acetylglycylglycine	44	L-Aspartic acid	110	Histamine, 10^{-2} M	28
DL-Alanine (pH 9.70)	159	Benzoylglycylglycine	24	Histamine, 10^{-3} M	107
DL-Alanine (pH 8.54)	154	Betaine	31	Histamine, 10^{-4} M	134
β-Alanine	96	Dimethylglycine, 10^{-2} M	142	Histidine, 10^{-2} M	4
γ-Aminobutyric acid	38	α,α′-Dipyridyl, 10^{-2} M	27	Histidine, 10^{-4} M	154
α-Amino-*n*-butyric acid	167	α,α′-Dipyridyl 10^{-4} M	150	Imidazole, 10^{-2} M	120
ε-Aminocaproic acid	39	β-Ethanolamine	136	Imidazole, 10^{-4} M	74
2-Amino-2-hydroxymethyl-1,3-propanediol	153	Ethylamine	51	Methionine	166
α-Aminoisobutyric acid	165	Glucosamine	141	Norleucine	173
Aminomethanesulfonic acid	106	Glutamic acid	169	KCN, 10^{-2} M	0
2-Amino-2-methyl-1,3-propanediol	149	Glycine	145	KCN, 10^{-4} M	161
α-Aminophenylacetic acid	147	Glycine anhydride (pH 9.76)	104	Proline	162
3-Aminopropanol	116	Glycine anhydride (pH 8.65)	27	Sarcosine	165
δ-Aminovaleric acid	38	Glycine ethyl ester (pH 9.67)	168	Taurine	99
		Glycine ethyl ester (pH 8.69)	133	Versene	0
		Glycylglycine	161	Control, 19 determinations	44 ± 14†

* The enzyme preparation had a specific activity of 6000 phosphatase units per mg. of protein nitrogen. The incubations were carried out in quartz tubes and the activity values are in terms of the phosphatase units per ml. calculated back to the stock enzyme solution.

† Standard deviation.

with two and three correctly positioned hydroxyl groups, respectively, were then tested and found to be efficient activators.[15] Since chelation ability appeared to be of primary importance in an activating molecule, the effects of KCN and α,α′-dipyridyl were also determined. Both of these compounds gave nearly optimal activation at a concentration of 10^{-4} M.

Analysis for Metals

Activation characteristics of our enzyme preparations were such as to indicate that metal ion interactions were involved. It then became of interest to ascertain which metals were present in our best preparations. Qualitative emission spectrographic observations are presented in Table II.[16] The preparation analyzed was that used to obtain the electrophoretic pattern of Fig. 1. Elements of major importance seem to be zinc, magnesium, and copper, in that order. The presence of magnesium was not unexpected. With respect to the presence of copper, it is of interest to note that the enzyme solution was pale green before and after dialysis against bicarbonate. This color changed to very pale yellow after dialysis against 0.1 M alanine which indicated that an appreciable amount of the copper had been removed. The effect of adding trace amounts of cupric ion to dilute enzyme solutions was then investigated (Fig. 3). The inactivating effect of the added cupric ion was nearly completely reversed by the subsequent addition of alanine.

Quantitative analysis of the specimen analyzed spectrographically revealed the presence of substantial amounts of zinc (Table III). It was also observed that 40 per cent of this zinc could be removed by dialysis against alanine, a process that greatly diminished the alanine activation of the preparation. The resulting material, containing 0.15 per cent of zinc, had a specific activity of over 10,000 units per mg. of protein nitrogen. Dialysis of this preparation against Versene (disodium salt of ethylenediaminetetraacetic acid, Dow Chemical Company) caused an inactivation that was accompanied by a proportional loss in zinc. Moreover, this inactivation by Versene occurred in the presence of excess magnesium, which indicated that the effect was not a result of loss of magnesium by the enzyme molecule.

To test further the possibility that swine kidney alkaline phosphatase was a zinc metalloenzyme, the increase in zinc content accompanying the increase in enzyme specific activity was determined (Fig. 4). A clear linear relation was obtained between enzyme purity and zinc concentration.

TABLE II

*Qualitative spectrographic analysis of alkaline phosphatase preparation**

Qualitative classification	Metals present in enzyme	
	After dialysis against 0.01 M $NaHCO_3$	After additional dialysis against 0.1 M alanine
Major	Zn, Mg, Cu, Al, Fe	Zn, Mg, Cu, Fe
Minor	Mn, Ca, Na, Pb, P	Mn, Ca, Na, Pb, P
Trace	Ni, Pt, Ag, Mo	Pt

* The elements are listed in very approximate order of decreasing occurrence, as judged by spectral line intensity. B, As, and Si were present in all of the samples. A specific search for Co failed to reveal the presence of this element. The solutions tested were dried on carbon electrodes and analyzed without preliminary ashing. The water and reagents were of the highest purity obtainable and were purified further by redistillation or recrystallization.

[15] Use of these compounds as buffers in investigations of alkaline phosphatase precludes studies of amino acid activation because of the activating effect of the buffer itself.

[16] The spectrographic data was obtained through the cooperation of LeRoy S. Brooks and E. Runge of the Ford Motor Company.

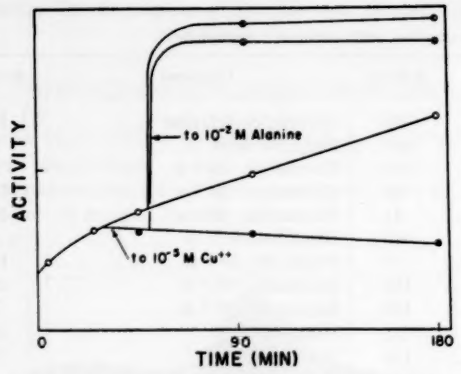

FIG. 3. Effects of cupric ion and alanine on the activation of alkaline phosphatase. The enzyme preparation had a specific activity of 6000 protein units per mg. of protein nitrogen. Conditions were those of the routine preliminary activation except for the addition of $CuSO_4$, the altered timing of the addition of the alanine, and the use of specially purified reagents and quartz tubes.

TABLE III

*Alteration of zinc content and activity of alkaline phosphatase by dialysis**

Dialysis against	0.01 M $NaHCO_3$	0.1 M Alanine	0.01 M Versene	0.02 M $MgSO_4$ also 0.01 M in Versene
Zinc (%)	0.26	0.15	0.01	0.025
PU/mg. PN†	8550	10440	1020	2700

* The pH of the dialysis solutions was adjusted to approximately 8 with 0.2 M Na_2CO_3 (recrystallized). Versene was removed before analysis by dialysis against dilute $NaHCO_3$.

† Phosphatase units per mg. of protein nitrogen.

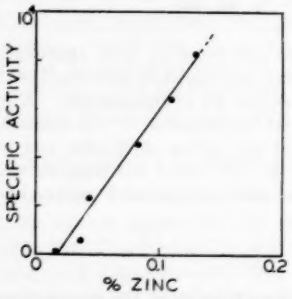

FIG. 4. Relation of specific activity of alkaline phosphatase to zinc content. The three preparations with specific activities over 2000 were dialyzed against 0.01 M alanine in 0.1 ionic strength phosphate buffer of pH 7.2. Specific activity is expressed in phosphatase units per mg. of protein nitrogen.

DISCUSSION

Purification—The specific activity of our best preparations seems to be higher than that of the intestinal preparations described by Morton (12), Roche and Bouchilloux (13), and Schramm and Armbruster (14), and the renal preparation discussed by Binkley *et al.* (15). Calculations based on the relative size of the major peaks in each of the two ultracentrifuge patterns and the specific activities of these preparations give values in reasonable agreement with the assumption that a pure enzyme would have a specific activity of 10,000 to 11,000 units per mg. of protein nitrogen. Additional support for this assumption is provided by the nature of the electrophoretic pattern and the fact that at no time has it been possible to prepare material with activities in excess of this figure. Thus, treatment of the enzyme with an activity of 10,000 units per mg. of protein nitrogen with $CHCl_3$, destroyed enzymic activity at the same rate that protein was denatured. Furthermore, exhaustive fractionation of material with a specific activity of 8000 units by electrophoresis-convection gave activities that reached a plateau in the 10,500 range.

Much the same problem was encountered in our work on alkaline phosphatase as has been reported by other investigators of this enzyme. The amount of the final, highly active preparation obtained was quite limited despite the fact that 8 kg. of cortex were processed every week for several years. It can be calculated that not more than 0.003 per cent of the protein of swine kidneys is alkaline phosphatase. The illusion that this tissue is an abundant source of the enzyme stems from the turnover number of approximately 100,000 moles per minute per 100,000 gm. of protein and from the sensitivity of the assay procedure.

Amino Acid Activation—Amino acid activation has been investigated a number of times since the first observations of Bodansky (16). Abuhl-Fadl and King (17) obtained results which indicated that amino acids did not activate kidney alkaline phosphatase. Akamatsu *et al.* (18, 19) observed that the enzyme was efficiently activated by all amino acids. Schales and Mann (20), on the other hand, obtained activation after simple alkaline incubation. Roche *et al.* (21) and Fischer and Greep (22), among others, have observed activation by amino acids.

We have observed that our preparations are activated not only by amino acids but by a number of other compounds. Our findings with amino alcohols parallel those of Granger and Fraux (23), and our observations on the effects of cyanide are in accord with those of Aso (24). When the structural requirements of an activating molecule are considered closely, it seems that ability to enter into complex formation with metal ions is of primary importance. Proper chelation strength seems necessary, since the powerful metal binding agent, Versene, inactivated the enzyme at all concentrations tested, and excessive amounts of KCN, histidine, and histamine were also inhibitory.

Metals and Enzyme Activity—The literature contains numerous references to metals and alkaline phosphatase (17, 25–33). For the most part, the findings described are based on the effects of various inhibitors and activators. In discussing the significance of such evidence Vallee (34) concludes that such studies are of restricted utility in the absence of direct analytical data. The present investigation provides evidence based on direct analysis to amplify and support inferences from data obtained with activators and inhibitors.

Our preparations of alkaline phosphatase contain approximately 0.15 per cent of firmly bound zinc, which seems to be essential for the activity of the enzyme. On the assumption that pure enzyme contains 0.17 to 0.18 per cent of zinc, a minimal molecular weight of approximately 37,000 can be calculated. Diffusion (35) and ultracentrifuge measurements (s_{20} = 6 to 6.5) indicate an intermediate molecular weight; therefore it is probable that one molecule of the enzyme contains several atoms of firmly bound zinc.

With respect to the relation of the metal content to the amino acid activation characteristics of the enzyme, the evidence im-

plicates both zinc and copper. It is probable that variable amounts of an inhibitory excess of zinc are present in the form of a loosely bound zinc-enzyme complex. The portion of the metal so involved may be removed by treatment with amino acids, and thus it can be differentiated from the more tightly bound, intrinsic zinc. The copper would appear to be exclusively inhibitory in nature.

It is generally accepted that magnesium is essential for alkaline phosphatase activity, and consequently it is probable that two metals are involved in the action of this enzyme. It is therefore of interest to note that under standard conditions of assay, the optimal concentration of magnesium is 5×10^{-3} M whereas the actual enzyme concentration is of the order of 10^{-8} M or less. In general, the magnesium concentration required for optimal activation approximates that of the substrate and leads one to speculate on a relation between this fact and the observations of Bamann and Nowotny (36) that certain metal ions, when combined with phosphoric acid esters, have a phosphatase-like effect. It may well be that the actual substrate for the enzyme is the magnesium salt of the phosphate ester under attack.

SUMMARY

Details are presented for a method of isolation of the alkaline phosphatase of swine kidneys with which a purification of approximately 10,000-fold has been obtained. The specific activity of the enzyme was increased from 1 to 2 units per mg. of total nitrogen in the source tissue to 10,500 units per mg. of protein nitrogen. The material obtained consisted of 80 to 90 per cent of one component by analysis in the ultracentrifuge and by free electrophoresis.

The activation characteristics of the enzyme were studied with a wide variety of amino acids, peptides, amino acid derivatives, and analogous compounds, and it was observed that the structural requirements for an activating molecule were very similar to the structure required of organic compounds for the formation of chelates with metal ions. It was concluded that amino acid activation of alkaline phosphatase results from complex formation with inactivating or inhibiting ions, or with both.

Qualitative spectroscopic analysis of a preparation of the enzyme revealed the presence of significant amounts of zinc, magnesium, and copper. The zinc content of this preparation was found to be 0.26 per cent. This value could be reduced to 0.15 per cent by dialysis against alanine, and the resulting enzyme was fully active without alanine activation. Removal of any part of the remaining zinc by dialysis against Versene or magnesium Versenate resulted in directly proportional losses in enzymic activity. A direct proportionality was also demonstrated between increases in specific activity and zinc content of the enzyme during the course of purification.

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Preparation and Some Properties of a Phosphate-activated Glutaminase from Kidneys*

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(Received for publication, June 26, 1958)

Glutamine participates in such diverse processes as urinary ammonia formation, transamination reactions, and biosynthesis of glucosamine, proteins, and purines (*cf.* (1) for pertinent references). The amide group of glutamine can be liberated as ammonia by two types of enzymes. One catalyzes the hydrolysis of glutamine to glutamic acid and ammonia and is dependent upon the presence of phosphate ion (or arsenate ion) (2, 3). The other catalyzes the transamination of glutamine with keto acids with the formation of α-ketoglutaramic acid, which is subsequently deamidated by an ω-amidase to give ammonia and α-ketoglutaric acid (4). The transamination and deamidation enzymes have been separated and their properties described (5, 6). Progress in the study of the phosphate-activated glutaminase has been slow because of the difficulties encountered in extracting and stabilizing this enzyme, which has only recently been obtained in a soluble form (7). A 40- to 80-fold purification of the enzyme has been reported (8). In the present study, some properties of the soluble enzyme prepared from dog kidney cortex are reported, and a relatively simple procedure is described by which 100- to 400-fold purification can be achieved.

EXPERIMENTAL

Materials and Methods

Source of Enzyme—Dog kidneys were frozen immediately after removal and stored. Extraction of the enzyme was greatly enhanced by prolonged storage in the frozen state. Kidneys routinely were kept frozen for at least a month before use.

Analytical Methods—Ammonia determinations were made by a diffusion procedure. Aliquots of the deproteinized samples were added to 20-ml. serum bottles which contained 1 ml. of saturated potassium carbonate. The bottles were stoppered with rubber serum bottle stoppers in which were inserted ground glass rods dipped into 1 N sulfuric acid. Diffusion of ammonia was allowed to take place over a period of 1 hour while the bottles were rotated on a large wheel. After diffusion was complete, the ammonia that was trapped as ammonium sulfate on the glass rods was washed into optically matched colorimeter tubes. 2 ml. of diluted Nessler's reagent (Folin) were added, the volume was brought to 10 ml., and readings were made in a Weston colorimeter with the 445 mμ filter. Quantitative recoveries of added ammonia were achieved by this method.

Protein concentrations were estimated usually by the method of Warburg and Christian (9), according to the formula of Kalckar (10). The results were found to be in excellent agreement with those obtained by the procedure of Lowry *et al.* (11).

Glutaminase Assay—Routine enzyme incubations proceeded for 15 minutes at 37° in a Dubnoff metabolic shaking incubator at a final volume of 2 ml. in 20 ml. beakers in 0.1 M phosphate buffer, pH 8.1. In many of the experiments to be described these conditions were varied as required. The reaction was stopped and the protein precipitated with 2 ml. of 25 per cent trichloroacetic acid. After centrifugation, aliquots of the supernatant fluid were assayed for ammonia content. Suitable enzyme and substrate blanks were subtracted.

Unit of Enzymatic Activity—The unit of activity was defined as the amount of enzyme which catalyzed the liberation of 1 μg. of ammonia in 15 minutes.

Fractionation Procedure—Frozen dog kidneys were allowed to thaw at room temperature until they could be dissected easily. All subsequent operations were carried out with cold solutions and in a refrigerated centrifuge. Only the cortex was used for enzyme preparation. The cortices were homogenized in a Waring Blendor for 4 minutes with 4 volumes of 0.1 M phosphate buffer, pH 8.1. The first 2 minutes were at reduced but gradually increasing speeds, and the final 2 minutes were at full speed. The homogenate then was centrifuged in the No. 30 head of the Spinco model L ultracentrifuge for 30 minutes at 30,000 r.p.m. (80,000 $\times$ *g*, average), and the sediment was discarded. The supernatant solution usually had approximately the same activity per unit volume as the original homogenate. The supernatant fluid was made 0.5 M with respect to sodium sulfate by the addition of the solid salt with stirring. After standing for 15 minutes, the material was centrifuged for 30 minutes at 30,000 r.p.m. in the No. 30 rotor of the Spinco ultracentrifuge. The precipitate was discarded, and the concentration of sodium sulfate in the clear supernatant fluid was increased to 1.0 M. After 15 minutes, the precipitate containing the enzyme was removed by centrifugation as above and taken up in 0.1 M phosphate buffer, pH 8.1. The supernatant fluid was discarded.

Salt fractionation was repeated several times, each time with precipitation of the enzyme within a slightly narrower range of salt concentrations. The final precipitate was collected between 0.55 and 0.65 M sodium sulfate. Preparations of highest specific activity were usually obtained after 5 successive salt fractionations. The ratio of optical density at 280 to 260 mμ approached

* This investigation was supported by a research grant (No. C 2568) from the National Cancer Institute, National Institutes of Health. A preliminary report of this work was presented at the forty-ninth annual meeting of the American Society of Biological Chemists in Philadelphia in April, 1958 (*Federation Proc.*, **17**, 303 (1958)).

TABLE I

Fractionation of dog kidney glutaminase

A representative fractionation of the phosphate-activated glutaminase from dog kidney cortex is presented. Original homogenate consisted of a 1:4 ratio of homogenate of cortices in 0.1 M phosphate buffer, pH 8.1; supernatant fluid A, was the supernatant obtained from centrifuging 30,000 r.p.m. (average 80,000 × *g*) in the No. 30 head of the Spinco model L ultracentrifuge. P-1, 1.0 M represents the first sodium sulfate fractionation, with enzyme precipitated between 0.5 and 1.0 M sodium sulfate; P-2, 0.8 M, the second sodium sulfate fractionation, with enzyme precipitated between 0.5 and 0.8 M sodium sulfate concentrations; P-3, 0.7 M, the third fractionation, with enzyme precipitated between 0.5 and 0.7 M sodium sulfate; P-4, 0.65 M, the fourth fractionation, with enzyme precipitated between 0.5 and 0.65 M sodium sulfate; and P-5, 0.65 M, the fifth fractionation, with enzyme precipitated between 0.55 and 0.65 M sodium sulfate concentrations. The optical density ratio at 280 to 260 mμ approached 1.6 by P-3, 0.7 M.

Preparation	Specific activity	Purification	Total units recovered
	μg.NH₃/mg.		
Homogenate	5.7	1	181,400
Supernatant fluid A	14.7	2.6	134,800
P-1, 1.0 M	134	23.4	114,000
P-2, 0.8 M	220	38.3	66,000
P-3, 0.7 M	385	67.2	37,400
P-4, 0.65 M	707	123	29,200
P-5, 0.65 M	1,590	277	7,100

1.6 after the third fractionation and changed very little subsequently, despite significant increases in specific activity. A 400-fold purification over the starting material was obtained in the best preparations. The most highly purified fractions lost 75 per cent of the activity within a week. Less highly purified preparations retained activity for considerably longer periods of time. Details of a typical fractionation are shown in Table I.

Other conventional methods of enzyme fractionation were unsatisfactory because of inactivation or low yield.

RESULTS

pH Activity Curve—Fig. 1 shows enzymatic activity as a function of pH. Optimal activity was obtained in the range from pH 7.9 to 8.1, a value in accord with other reports for renal glutaminase (12). Crude human placental glutaminase showed a pH optimum between 8 and 10 (13). The activity decreased rapidly and irreversibly at pH values of 7.0 or below. This is in sharp contrast to the bacterial glutaminase of *Clostridium welchii* which possesses optimal activity at pH 5 (14).

Effect of Temperature—A study of enzymatic activity as a function of temperature showed maximal activity at 40°, under the conditions of assay. The energy of activation as determined by the Arrhenius equation for the 30–40° range was found to be 8250 calories per mole. The heat of inactivation, as determined by measurement of residual activity after heating a solution of the enzyme for 5 minutes at different temperatures, was estimated to be approximately 37,500 calories per mole.

Effect of Enzyme Concentration—The activity observed was linear with regard to enzyme concentration over a 10-fold range.

Time Course of Reaction—The reaction was linear with time up to about 60 minutes. Quantitative determination of glutamine can be readily accomplished by these preparations when an excess of enzyme is used.

Effect of Substrate Concentration—The initial reaction rates for the deamidation of glutamine were measured at different substrate concentrations. From these data, the Michaelis-Menten constant for the reaction has been determined to be 5×10^{-3} moles per l. from a Lineweaver-Burk (15) plot.

Substrate Specificity—The purified enzyme deamidated glutamine, but not asparagine, nor a variety of amides, nor several glutamine analogues (Table II). α-Methylglutamine and isoglutamine were decomposed at a slower rate than glutamine itself. The lowered activity when glutamine and isoglutamine

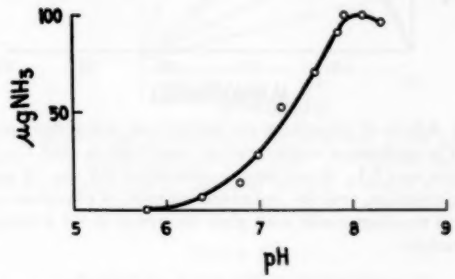


FIG. 1. Effect of pH on glutaminase activity. 0.1 M phosphate buffer at specified pH.

TABLE II

Action of glutaminase on various substrates

Compounds were tested at 0.025 M concentration in 0.1 M phosphate buffer, pH 8.1, under the conditions described in the text under "Glutaminase Assay." Incubation mixtures were analyzed for ammonia in the case of the amides, hydrazine for γ-L-glutamylhydrazide, or glutamic acid for L-glutamic-γ-methyl ester, γ-L-glutamylmethylamide, and γ-DL-glutamylbutylamide.

Substrate	Activity
L-Glutamine	100
L-Isoglutamine + L-glutamine*	88
L-Isoglutamine†	34
α-Methyl-DL-glutamine†	24
γ-L-Glutamylhydrazide‡	5
L-Glutamic-γ-methyl ester†	0
L-Asparagine	0
α-Methyl-DL-asparagine†	0
γ-L-Glutamylmethylamide§	0
γ-DL-Glutamylbutylamide†§	0
Nicotinamide	0
Acetamide	0
Adenine	0

* 0.025 M concentration of both substances.

† Samples of L-isoglutamine were kindly supplied by Dr. Alton Meister of Tufts University and Dr. Fredrick Carpenter of the University of California. α-Methyl-DL-glutamine, γ-L-glutamylhydrazide, L-glutamic-γ-methyl ester, α-methyl-DL-asparagine, and γ-DL-glutamylbutylamide were a gift of Dr. Karl Pfister of Merck and Company, Inc. γ-L-Glutamylmethylamide was kindly supplied by Dr. F. C. Meyer of Monsanto Chemical Company.

‡ Hydrazine measured by Lutwack's method (16).

§ Glutamic acid estimated by a chromatographic procedure.

were mixed in isomolar amounts suggests that the latter compound may be a competitive substrate. The γ-methyl ester of L-glutamic acid was not acted upon; it inhibited to the extent of 64 per cent when present in isomolar amounts with glutamine. γ-L-Glutamylmethylamide, although not acted upon itself, consistently enhanced activity on glutamine by approximately 20 per cent when present in isomolar amounts with glutamine. γ-DL-Glutamylbutylamide did not show this enhancement of activity. A slow liberation of hydrazine from γ-glutamylhydrazide occurred. When hydroxylamine was present during the action of the enzyme on glutamine, hydroxamate formation could not be detected by the method of Lipmann and Tuttle (17), nor was it possible to demonstrate a transfer function for the enzyme in several other test systems.

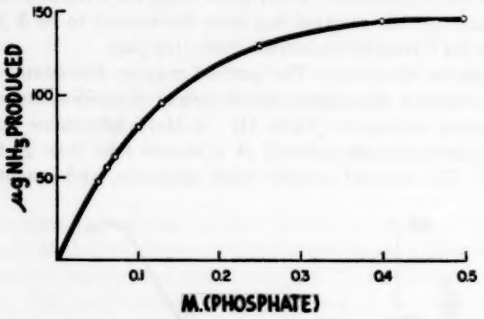


FIG. 2. Effect of phosphate on activity of dog kidney glutaminase. The glutamine concentration was 0.025 M, and the pH of all samples was 8.1. Each sample contained 0.6 mg. of protein, 0.025 M glutamine, and the indicated amount of phosphate at pH 8.1 (all pH readings made with glass electrode at 0.1 M phosphate concentration).

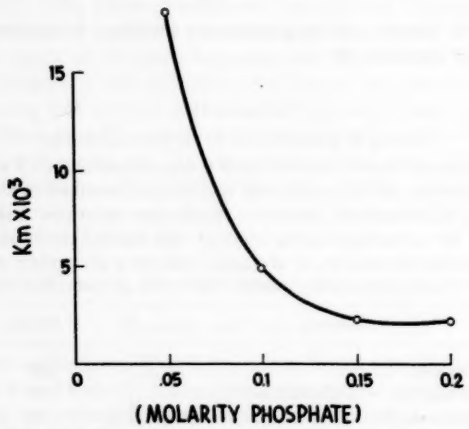


FIG. 3. Effect of phosphate concentration on Michaelis constants

Effect of Anions—Glutaminase has been reported to be activated by phosphate, arsenate, and sulfate ions, with phosphate reported as the most effective (2). These results have been confirmed. Arsenate and sulfate were observed to activate in the presence of suboptimal phosphate concentrations, but inhibited in the presence of phosphate at optimal concentration. Sodium chloride, bromide, and cyanide, which have been found to activate bacterial glutaminase (14), inhibited the kidney enzyme in the presence of phosphate.

The effect of phosphate on glutaminase activity is shown in Fig. 2. The findings are in agreement with previous reports in which the optimal molar ratio of phosphate to glutamine was estimated to be approximately 17. A plot of values obtained from Lineweaver-Burk plots of results of experiments performed at different phosphate concentrations (Fig. 3) indicates that the affinity of enzyme for substrate is increased with increasing concentrations of phosphate up to approximately 0.2 M.

Effect of Inhibitors—The action of a number of inhibitors has been studied in an attempt to determine the characteristics of the active site of glutaminase. Three types of inhibition of the enzyme have been observed; sulfhydryl, competitive with glutamine, and competitive with phosphate.

The sulfhydryl reagents, mercuric chloride, *p*-chloromercuribenzoate, iodoacetamide, *N*-ethylmaleimide, and *p*-benzoquinone were found to be potent inhibitors of glutaminase. Inhibition by mercuric chloride, *p*-chloromercuribenzoate, and quinone was prevented by reduced glutathione or cysteine, whereas the effects of *N*-ethylmaleimide and iodoacetamide were not. Quinone had been previously reported to inhibit glutaminase (18), al-

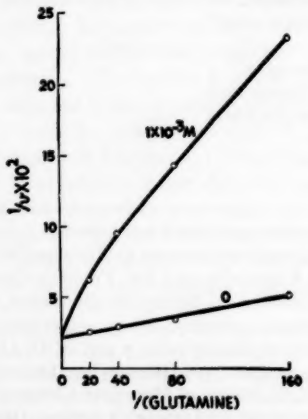


FIG. 4. Lineweaver-Burk plot of N-ethylmaleimide inhibition as a function of glutamine concentration. The phosphate buffer concentration was 0.1 M at pH 8.1.

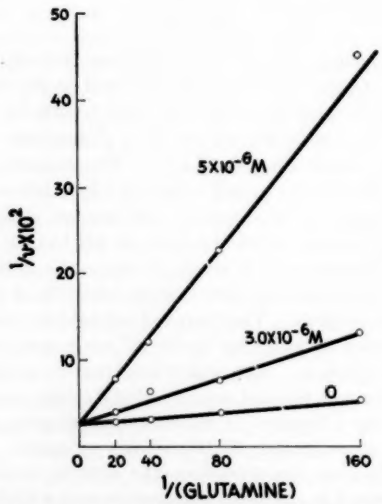


FIG. 5. Lineweaver-Burk plot of *p*-chloromercuribenzoate inhibition as a function of glutamine concentration. The phosphate buffer concentration was 0.1 M at pH 8.1.

though no evidence for its action as a sulfhydryl inhibitor was presented. A Lineweaver-Burk plot (Fig. 4) of inhibition by *N*-ethylmaleimide yielded a curve which showed inhibition to be more nearly competitive with glutamine than noncompetitive, whereas the results with *p*-chloromercuribenzoate gave Lineweaver-Burk plots typical of competitive inhibition (Fig. 5). Inhibition by *p*-chloromercuribenzoate was shown to be independent of phosphate concentration. From these data it seems probable that a sulfhydryl group of the enzyme is a site of attachment for the substrate.

Ammonia Inhibition—Ammonia was found to inhibit glutaminase in a manner competitive with glutamine (Fig. 6).

Phthalein Inhibition—Glutaminase has been reported to be inhibited by a number of phthalein dyes. Bromocresol green (3,3′,5,5′-tetrabromo-*m*-cresol-sulfonphthalein) and bromosulfalein (disodium phenoltetrabromophthalein-disulfonate) were reported to inhibit the phosphate-activated glutaminase from *C. welchii* in a manner competitive with glutamine (14). These results were not confirmed with the preparations from dog kidney. Bromocresol green was found to be a noncompetitive inhibitor with respect to glutamine (Fig. 7), but when studied as a function of phosphate concentration, the Lineweaver-Burk plots were characteristic of competitive inhibition (Fig. 8). Similar data were obtained for bromosulfalein and flavianic acid (2,4-dinitro-1-naphthol-7-sulfonic acid). The relative effectiveness of these inhibitors can be seen in Table III. The differences in the type of inhibition produced by the phthaleins in the bacterial and mammalian kidney glutaminase may be a function of the pH of incubation or may be a result of fundamental differences in the enzymes obtained from these two sources.

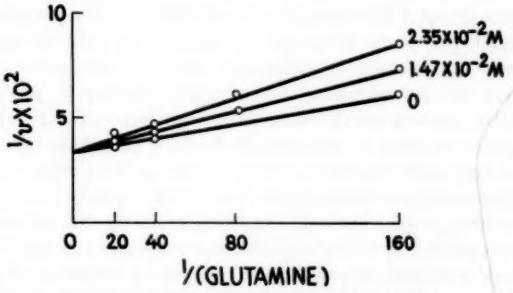


FIG. 6. Lineweaver-Burk plot of ammonia inhibition as a function of glutamine concentration. Reactions were run in 0.1 M phosphate buffer at pH 8.1.

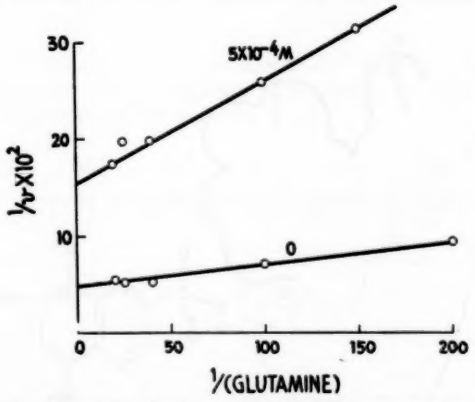


FIG. 7. Lineweaver-Burk plot of bromocresol green (tetrabromo-*m*-cresol-sulfonphthalein) inhibition as a function of glutamine concentration in 0.1 M phosphate buffer, pH 8.1.

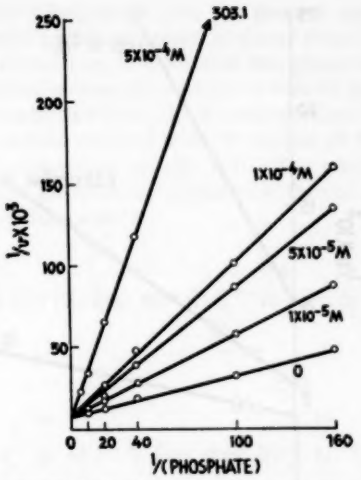


FIG. 8. Lineweaver-Burk plot of bromocresol green inhibition as a function of phosphate concentration. Glutamine was present at 0.025 M and phosphate buffer, at desired concentration at pH of 8.1.

TABLE III

Inhibitors of dog kidney glutaminase

Enzymatic activity was determined as described in the text in the presence of inhibitors at the specified concentrations.

Inhibitor added	Molar concentration of inhibitor	Inhibition
		%
Bromocresol green	1×10^{-3}	100
	5×10^{-4}	74
Bromosulfalein	1×10^{-3}	100
	5×10^{-5}	87
	1×10^{-5}	4
	1×10^{-6}	0
Bromocresol purple	1×10^{-3}	82
Flavianic acid	1×10^{-3}	79
	5×10^{-4}	68
	2.5×10^{-4}	45
Phenolphthalein	2×10^{-3}	0
p-Chloromercuribenzoate	1×10^{-3}	100
	5×10^{-4}	82
Mercuric chloride	1×10^{-3}	100
	5×10^{-4}	89
p-Benzoquinone	1×10^{-3}	92
N-ethylmaleimide	1×10^{-3}	100
	1×10^{-4}	50

Glutamic Acid Inhibition—Inhibition by glutamic acid was found to be noncompetitive with glutamine (Fig. 9) but com-

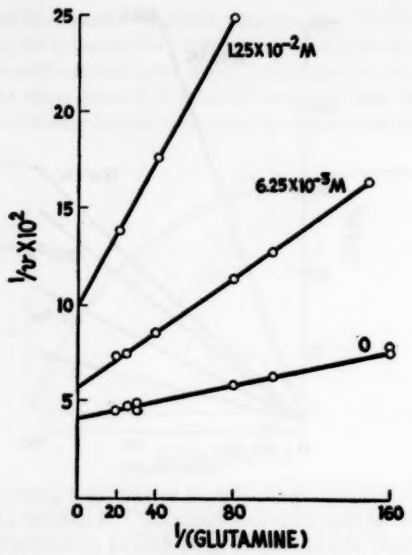


FIG. 9. Lineweaver-Burk plot of glutamic acid inhibition as a function of glutamine concentration. The phosphate buffer concentration was 0.1 M at pH 8.1.

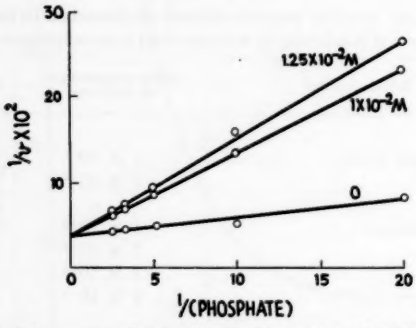


FIG. 10. Lineweaver-Burk plot of glutamic acid inhibition as a function of phosphate concentration. Glutamine was present at 0.025 M concentration. The pH of each sample, at a dilution of 0.1 M, was 8.1.

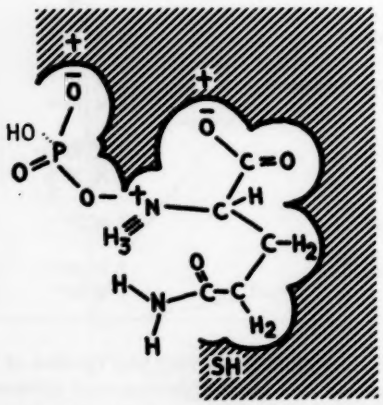


FIG. 11. Two-dimensional projection of scale models (Catalin) showing postulated attachment of glutamine to active sites of glutaminase (see the text).

petitive with phosphate (Fig. 10). Glutaric, α-ketoglutaric, γ-aminobutyric, and α-amino-ε-hydroxycaproic acids were not inhibitory in concentrations comparable to those found to be effective in the case of glutamic acid. It seems, therefore, that the α-amino and γ-carbonyl groups are necessary for inhibition.

DISCUSSION

The data presented provide a tentative explanation of the mechanism of glutaminase action. Glutaminase may be postulated to have a sulfhydryl and two cationic sites. One of the cationic sites may serve as a point of attachment for a phosphate ion, with the substrate attaching to the other cationic site through the α-carboxylate group and to the second negative charge of the phosphate group through a positively charged α-amino group. Such a mechanism would presume that glutamine could attach most readily to an active site already containing phosphate (or other suitable divalent anion). Hydrolysis of the amide would be expected to take place in the vicinity of the sulfhydryl group. A sketch made from the two-dimensional projections of scale models (Catalin models) illustrates this concept (Fig. 11).

The increasing affinity of substrate for enzyme with increasing levels of phosphate (Fig. 2) suggests that phosphate plays an important role in the attachment of substrate to enzyme. The high levels of phosphate ion required for maximal activity indicate that the affinity of phosphate for enzyme is relatively low, as would be expected if an ionic bond with a cationic site on the enzyme protein were involved. Ammonia, an inhibitor competitive with glutamine, might be expected to compete with the α-amino group of the substrate for attachment to the phosphate. Although phosphate is known to enhance the rate of nonenzymatic deamidation of glutamine, sufficient differences exist between the enzymatic and nonenzymatic reactions to preclude a similar function for the anion in the two processes (3). It is not known whether or not the high levels of phosphate required in the enzymatic studies can occur at intracellular sites of glutaminase action, or whether phosphate is substituting in the system *in vitro* for some naturally occurring, more effective activator.

Superposition of the two-dimensional projections of models of flavianic acid and bromosulfalein, two potent inhibitors of glutaminase, on the projection of the postulated active site revealed a good fit of the inhibitor molecules to the proposed site (Figs. 12 and 13). In bromosulfalein, the distance between two negatively charged oxygen atoms on the sulfonic acid groups, and in

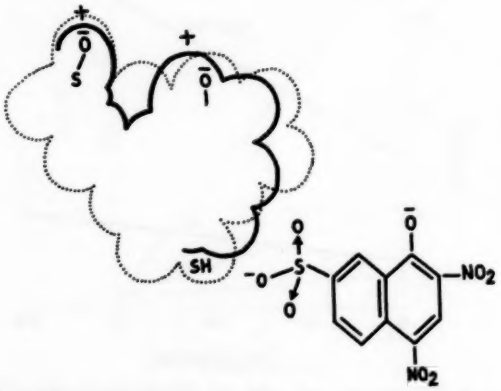


FIG. 12. Superposition of two-dimensional projection of flavianic acid model on the projection of the postulated active site.

flavianic acid, the distance between negatively charged oxygen atoms of sulfonic acid and phenolate groups approximate the distance between the cationic sites opposing the negative phosphate and carboxylate oxygen atoms of the phosphate-glutamine complex shown in Fig. 11. The finding that inhibitions by these substances were competitive with phosphate and not with glutamine is compatible with the hypothesis that the active site for attachment and subsequent hydrolysis of glutamine actually includes phosphate. Substances which compete with phosphate for attachment to the enzyme would inhibit enzyme activity, but their action could not be reversed by glutamine. Substances competitive with glutamine would then have to possess structures with affinity for the enzyme-phosphate complex. In a model of isoglutamine, a competitive substrate, the α-amino, γ-carboxyl, and α-amide groups can be made to lie in positions similar to those shown for the α-amino, α-carboxyl, and γ-amide groups, respectively, of glutamine.

Inhibition by *p*-chloromercuribenzoate seems to be competitive with glutamine which indicates that a sulfhydryl group is a point of attachment of substrate with enzyme. The participation of a sulfhydryl group in enzymatic deamidation might be envisioned by consideration of the possible interaction of two of three resonance hybrids of the amide structure with a sulfhydryl group (see Reaction 1). Structure A undoubtedly would be the most stable form and would give the greatest contribution. Although B has a greater separation of charge than C, it would probably be favored over C because of the greater number of covalent bonds and because the octet rule is violated by structure C. It might be expected that a significant proportion of the —SH groups on the enzyme would be in the S^- form at pH 8.1, the optimal pH for glutaminase activity (19). The reactions of B and C with the S^- groups of the enzyme could then be formulated as shown in Reactions 2 and 3.

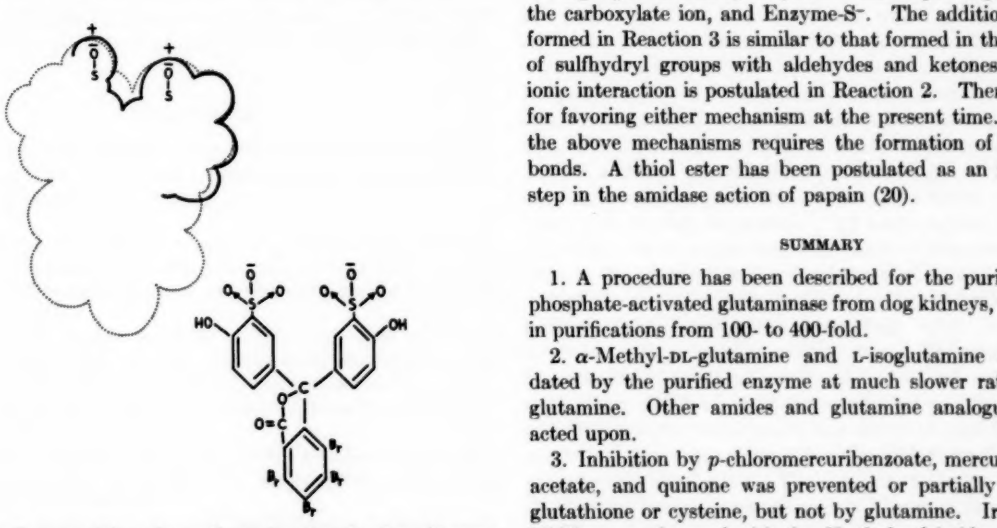
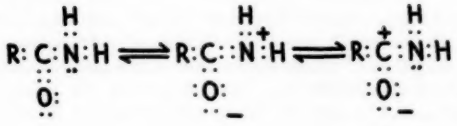
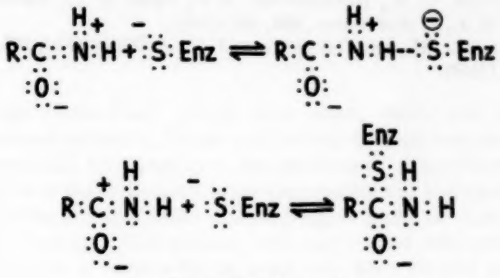


FIG. 13. Superposition of two-dimensional projection of bromosulfalein model on the projection of the postulated active site. The three rings are not coplanar.



A B C

REACTION 1



REACTIONS 2 AND 3

Both intermediates suggested in Reactions 2 and 3 above could undergo spontaneous hydrolysis at alkaline pH to give ammonia, the carboxylate ion, and Enzyme-S^-. The addition compound formed in Reaction 3 is similar to that formed in the interaction of sulfhydryl groups with aldehydes and ketones whereas an ionic interaction is postulated in Reaction 2. There is no basis for favoring either mechanism at the present time. Neither of the above mechanisms requires the formation of high energy bonds. A thiol ester has been postulated as an intermediary step in the amidase action of papain (20).

SUMMARY

1. A procedure has been described for the purification of a phosphate-activated glutaminase from dog kidneys, which results in purifications from 100- to 400-fold.

2. α-Methyl-DL-glutamine and L-isoglutamine were deamidated by the purified enzyme at much slower rates than was glutamine. Other amides and glutamine analogues were not acted upon.

3. Inhibition by *p*-chloromercuribenzoate, mercuric ion, iodoacetate, and quinone was prevented or partially reversed by glutathione or cysteine, but not by glutamine. Irreversible inhibition was observed with the *N*-ethylmaleimide.

4. Inhibition by ammonia was competitive with glutamine, whereas inhibition by glutamic acid, bromosulfalein, bromocresol green, or flavianic acid was found to be noncompetitive with glutamine but competitive with respect to phosphate.

5. A tentative mechanism of glutaminase action has been proposed.

Acknowledgment—The authors wish to express their gratitude to Dr. Georg Cronheim of Riker Laboratories for generous supplies of dog kidneys.

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The γ-Aminobutyric Acid-α-Ketoglutaric Acid Transaminase of Beef Brain*†

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(Received for publication, June 26, 1958)

γ-Aminobutyric acid was shown to be a constituent of the mammalian central nervous system by Roberts and Frankel (2) and by Awapara *et al.* (3). It was identified subsequently (4) as the main constituent of Florey's neurohumoral inhibitory Factor I (5). Some of the pertinent physiological and biochemical findings relating to the metabolism of this amino acid in the central nervous system have been summarized recently (6, 7).

ABA[1] is formed in brain tissues primarily by the decarboxylation of glutamic acid (8, 9). It can be transaminated reversibly with KGA to form glutamic acid and succinic semialdehyde in both brain and liver preparations of various mammalian species (10, 11). The following report describes some properties of the enzyme from beef brain that catalyzes the transamination reaction.

EXPERIMENTAL

All reagents were obtained from commercial sources[2] with the exception of ABA which was synthesized from 2-pyrrolidinone by the method of Tafel and Stern (12).

* This work was supported in part by grants from the Multiple Sclerosis Foundation, M.S. 101, and the National Institute for Neurological Diseases and Blindness, United States Public Health Service, No. 1615.

† Preliminary reports of parts of this work were presented before the Federation of American Scientists for Experimental Biology, Philadelphia, April 1958 (1), and before the Neurochemistry Section of the American Academy of Neurology, Philadelphia, April 1958.

[1] The abbreviations used are: ABA, γ-aminobutyric acid; KGA, α-ketoglutaric acid; ABA-T, γ-aminobutyric acid:α-ketoglutaric acid transaminase.

[2] Reagents were obtained from the following sources: pyridoxal-P (97 to 100 per cent purity), α-γ-diaminobutyric acid, α-ketobutyric acid, δ-aminovaleric acid, glycine, KGA, and glutamic acid from the California Corporation for Biochemical Research, Los Angeles, California; α-aminobutyric acid and sodium pyruvate from Nutritional Biochemicals Corporation, Cleveland, Ohio; β-alanine from General Biochemicals, Chagrin Falls, Ohio; oxaloacetic acid and *p*-chloromercuribenzoate from Sigma Chemical Company, St. Louis, Missouri; 2-pyrrolidinone from Cliffs Dow Chemical Company, Marquette, Michigan; iodoacetic acid amide from Mann Research Laboratories, New York, New York; ascorbic acid, 4-methoxymethyl pyridoxine, strychnine sulfate, Veronal and benactyzine hydrochloride from Merck Sharp and Dohme, Rahway, New Jersey; ε-amino-*n*-caproic acid, sodium malonate and hydroxylamine hydrochloride from Eastman Kodak Company, Rochester, New York; iproniazide from Hoffman La-Roche, Nutley, New Jersey; procaine hydrochloride and Diamox from American Cyanamid Company, New York; serotonin-creatine sulfate from Abbott Laboratories, North Chicago, Illinois; N-ethylmaleimide from Delta Chemical Works, New York, New York. The Dilantin was a gift from Dr. Keith Killam. All other chemicals were Baker analyzed reagents from J. T. Baker Chemical Company, Phillipsburg, New Jersey.

[3] Kindly donated by the Atlas Packing Company, Los Angeles, California.

Preparations—Fresh brains[3] from steers which had been slaughtered without a preliminary blow on the head were packed in crushed ice for transport to the laboratory. After dissecting out most of the white matter, the gray matter was homogenized in a 10-fold excess of Baker acetone (reagent grade) at −10° and filtered rapidly. The resulting cake was washed with acetone and dried in a vacuum for 24 hours over silica gel and in the presence of paraffin shavings. The powder was stored at −4° in a desiccator and retained most of its ABA-T activity over a period of 5 months.

Homogenates of the beef brain acetone powder were prepared in a small cup of the Waring Blendor with 1 gm. of powder, 10 ml. of 0.1 M borate buffer, pH 8.2, and a drop of 0.1 M sodium Versenate solution adjusted to pH 8.0 with sodium hydroxide. Enzyme solutions were prepared by centrifuging the homogenate in a Spinco model L ultracentrifuge at 100,000 × *g* for 30 minutes. The resulting precipitate, which was virtually devoid of ABA-T activity, was discarded. Every 5 ml. of supernatant, or an ammonium sulfate fraction prepared therefrom, was dialyzed with stirring, for a total of 2.5 hours against five changes of 500-ml. borate buffer and then used on the same day. All steps of the preparation were performed at approximately 4°.

Methods—Samples were incubated with substrates at 37° in rubber-capped, round-bottom centrifuge tubes in a Dubnoff incubator with a modified shaking tray. The reaction usually was carried out in a final volume of 1.4 ml. and was stopped by the addition of 0.6 ml. of 25 per cent trichloroacetic acid. After centrifugation the supernatant was decanted, and the trichloroacetic acid was removed by extraction with three 5-ml. portions of water-saturated ether. The residual ether was removed by shaking the tubes for approximately 1 minute in a water bath at 80°.

The method devised for the determination of the glutamic acid formed during the reaction is based upon the observation by Ley (13) that, under specific conditions, α-amino acids chelate copper strongly, whereas the chelation of γ-amino acids with copper is negligible. In mixtures containing dialyzed brain extracts, ABA, KGA, glutamic acid, and succinic semialdehyde (or succinate), only glutamic acid was found to chelate with copper under the conditions used. Copper-glutamic acid chelates were

made in the following manner: 3 ml. of a copper phosphate slurry prepared according to directions given by Albanese and Irby (14) were added to a 1-ml. aliquot of the protein-free supernatant of the incubation mixture. After 10 minutes, the mixture was centrifuged to remove the excess slurry. The amount of copper-glutamic acid chelate in solution was measured with Neocuproine (G. Frederick Smith Chemical Company), a phenanthroline reagent suitable for the colorimetric determination of very low concentrations of copper (15). Solutions of 0.0069 M Neocuproine in acetate buffer, pH 5.0, were prepared (16). 3 ml. of the cold Neocuproine reagent were added to a 1-ml. aliquot of the supernatant containing the copper-glutamic acid chelate. After the addition of at least 50 mg. of crystalline ascorbic acid, the yellow color of the cuprous-Neocuproine complex was allowed to develop at room temperature over a period of at least 10 minutes and was then read at 454 mμ in a Beckman model DU spectrophotometer. Readings obtained for the experimental samples were corrected by subtracting values found in tubes deproteinized at zero time. Virtually identical blank values were obtained for incubated tubes in which either ABA or KGA were omitted. Water was substituted for glutamic acid to give a reagent blank, and two glutamic acid standards were included with each experiment.

The amount of copper determined by this method was proportional to the glutamic acid in the incubation mixture (Fig. 1). Up to 7 μmoles of glutamic acid in the original incubation mixture could be measured as described above; duplicate determinations checked to within 0.1 μmole. Occasionally larger amounts of the acid were measured by reducing the size of the sample aliquots. In several instances, determinations of the acid by the above method and by the decarboxylase procedure (17) were compared and gave concordant results. Protein determinations were made by the method of Lowry *et al.* (18).

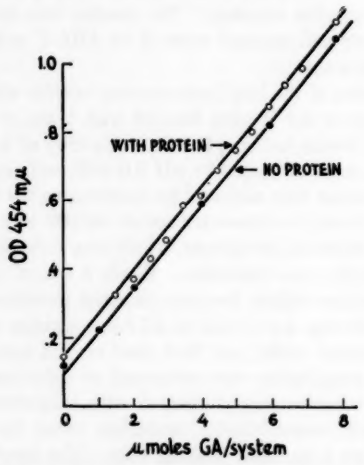

FIG. 1. The relationship of optical density to glutamic acid content in the incubation system using the Neocuproine method, as outlined in "Experimental" for the determination of glutamic acid.

TABLE I

Activity of ABA-T in acetone powder extracts from various sources

Samples were incubated in borate buffer at pH 8.1 in the standard 1.4-ml. system containing 40 μmoles of KGA, 40 μmoles of ABA, and enzyme preparation. Because of technical difficulties separation of gray and white matter was not complete.

Source	Glutamic acid formed per hour	
	per 100 mg. of acetone powder	per mg. of protein*
	μmoles	*μmoles*
Mouse brain	13.5	0.45
Beef brain (cortex)	7.4	0.17
Beef brain (white matter)	2.5	0.085
Escherichia coli strain E. 26	16.3	2.3

* Protein was determined according to the method of Lowry (18).

RESULTS

Occurrence—A survey of ABA-T activity in acetone powders from a number of sources is shown in Table I. The gray matter of beef brain was a far richer source of the enzyme than the white matter. However, the ABA-T activity of beef brain gray matter was low when compared to the activity found in preparations of whole mouse brain and acetate-adapted *Escherichia coli* strain E-26.

pH Optimum—The pH optimum for the ABA-T enzyme from beef brain was found to be 8.2 (Fig. 2). This value is similar to that reported for the ABA-T of mouse brain (11). In addition to borate buffer, ABA-T of beef brain also was active in phosphate, Veronal and tris(hydroxymethyl)aminomethane buffers. The tris(hydroxymethyl)aminomethane buffer was not used in our experiments because it chelated with copper. The enzyme retained activity when stored for less than an hour above the optional pH, but deteriorated rapidly and irreversibly under slightly acid conditions.

Reaction Velocity as a Function of Substrate and Enzyme Concentrations, Time of Incubation, and Temperature—Results of an experiment in which reaction velocity was measured as a function of the concentration of both substrates is shown in Fig. 3. Maximal activity of the preparation was attained at concentrations of 0.02 to 0.04 M for both KGA and ABA. The Michaelis-Menten constants, as calculated for a two substrate system (19, 20), were 3×10^{-3} M for ABA and 4×10^{-3} M for KGA, thus indicating a rather low affinity of the enzyme for the substrates. When assays were performed in the presence of optimal levels of substrates, activity was proportional to the amount of enzyme employed and the reaction was of zero order.

The maximal activity, for a 30-minute incubation period, was attained at an incubation temperature of 49° (Fig. 4).

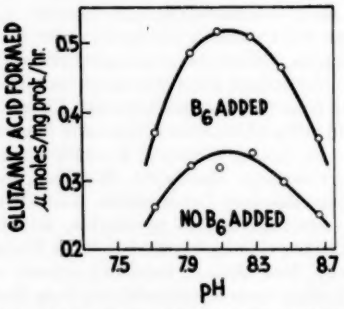

FIG. 2. Effect of pH and pyridoxal-P on reaction rate. Standard system with 40 μmoles of KGA, 40 μmoles of ABA, with and without 60 μg. of pyridoxal-P; 2-hour incubation.

Cofactor Requirement—Activation of ABA-T by pyridoxal phosphate at different pH levels was shown in Fig. 2. The largest increment in activity occurred at the optimal pH. The ABA-T activity of individual preparations measured at pH 8.2 could be enhanced from 20 to 130 per cent depending upon the procedures used during the preparation of the powders (21). Although low levels of pyridoxal-P markedly stimulated ABA-T activity, surprisingly high concentrations were required for maximal activation (Fig. 5).

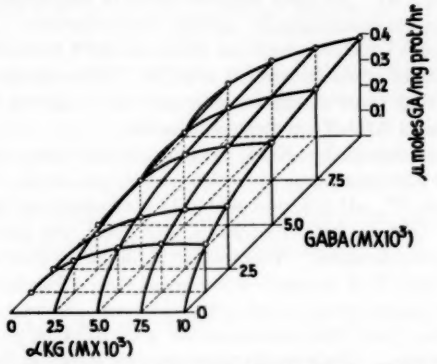

FIG. 3. Effect of substrate concentration on reaction rate, with suboptimal amounts of both substrates. The incubation was at 37°, pH 8.2, for 2 hours.

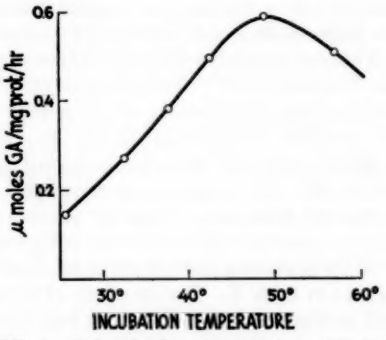

FIG. 4. Effect of incubation temperature on reaction rate. Standard system with 40 μmoles of KGA, 40 μmoles of ABA, 80 μg. of pyridoxal-P and enzyme, incubated at pH 8.2 for 30 minutes at temperatures indicated. 2 minutes were allowed for the tubes to reach the temperature of the incubation bath.

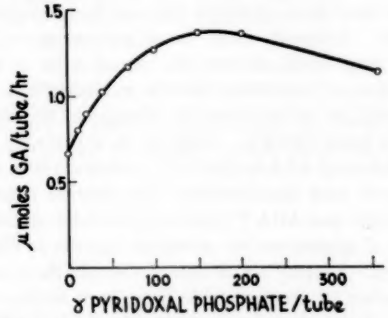

FIG. 5. Effect of pyridoxal-P concentration on reaction rate. Standard system with 2.8×10^{-2} M substrate concentration and pyridoxal-P additions as indicated. The amount of cofactor required for maximal activity varied from one batch of pyridoxal-P to another. The above experiment was conducted with a cofactor preparation of less than average activity.

TABLE II

Effect of some inhibitors on ABA-T

Samples were incubated in the standard 1.4-ml. system for 2 hours. This contained 40 μmoles of KGA, 40 μmoles of ABA, 80 μg. of pyridoxal-P, and enzyme preparation. All ingredients were buffered at pH 8.2 in borate buffer. Pyridoxal-P and enzyme were mixed before adding them to the system. Inhibitors were added last. There was no preincubation period, but samples were given 2 minutes to reach the temperature of the incubator bath. Zero time control samples were taken for each sample incubated.

Inhibitor	Concentration	Inhibition
	M	%
Semicarbazide HCl	1×10^{-2}	0
Hydroxylamine	1×10^{-4}	100
Pyridoxaloxime	1×10^{-4}	31
p-Chloromercuribenzoate	7×10^{-4}	100
N-ethylmaleimide	1×10^{-3}	93
	1×10^{-4}	15
Iodoacetic acid amide	1×10^{-3}	34
	1×10^{-4}	0

TABLE III

Hydroxylamine inhibition and reactivation by pyridoxal phosphate

Preparations I and II represent two different enzyme preparations. I was dialyzed *before* the addition of hydroxylamine, and II was dialyzed *after* the addition of hydroxylamine. For both preparations 5 ml. of enzyme were dialyzed against six 500-ml. changes of borate buffer at 3° over a period of 2.5 hours. The incubation was in standard systems as described in the text.

Preparation	Pretreatment NH_2OH concentration	Pyridoxal-P added μg./ml. incubation mixture	ABA-T activity (μmoles of glutamic acid /mg. of protein/hr.)
	M		
I		0	0.20
		60	0.34
		150	0.34
	1×10^{-4}	0	0
	1×10^{-4}	60	0.18
	1×10^{-4}	150	0.20
II		0	0.21
		60	0.35
	1×10^{-2}	0	0.06
	1×10^{-2}	60	0.20

Inhibitor Studies—ABA-T was inhibited by hydroxylamine at very low levels but not by semicarbazide even at 1×10^{-2} M concentration (Table II). Hydroxylamine inhibition was partially reversed by the addition of pyridoxal-P to the system. Extensive dialysis did not reactivate appreciably the enzyme inhibited by hydroxylamine, and the subsequent extent of activation by pyridoxal-P was similar to that found with the uninhibited enzyme (Table III).

The enzyme was inhibited by iodoacetic acid amide, *p*-chloromercuribenzoate and *N*-ethylmaleimide (Table II), suggesting that a free sulhydryl group may be necessary for the functioning of the transaminase.

Anaerobiosis, fluoride, arsenite, azide, cyanide and malonate,

TABLE IV

Transamination with KGA in beef brain acetone powder extracts

Incubations for 90 minutes in the standard system at 37° and pH 8.2. Substrate concentrations for KGA and the amino group donors was 2.8×10^{-2} M. For tests with substrates which themselves chelated copper, the glutamic acid formed was separated by one-dimensional paper chromatography (phenol-H_2O) and measured by a ninhydrin method (26). Samples were run in triplicate.

Amino group donor	Relative activity
	%
ABA	100
β-Alanine	96
δ-Aminovaleric acid	46
ε-Aminocaproic acid	0
Glycine	10†
α-Aminobutyric acid	80†
α,γ-Diaminobutyric acid	13†
α,γ-Diaminoglutaric acid*	245†

* The α,γ-diaminoglutaric acid used in these experiments was prepared by the method of Carter (27).

† Chromatographic isolation.

TABLE V

Protection of ABA-T by substrates and cofactor during preincubation at different temperatures

Tubes reached preincubation temperature in 1 minute and were then preincubated for an additional 3 minutes. At the end of this period, the tubes were placed at 0° and the appropriate additions of substrates and cofactors were made. Subsequently, the tubes were incubated for 2 hours at 37° as described in the text. Substrate concentrations during the preincubation period were 1.3×10^{-2} M and 50 μg. of cofactor were added whenever indicated. For the final incubation at 37° for 2 hours, substrate concentrations were adjusted to 3×10^{-2} M and 150 μg. of pyridoxal-P per tube. High levels of pyridoxal-P were added to avoid errors caused by possible pyridoxal-P destruction during preincubation at pH 8.2.

Additions before preincubation	ABA-T activity*		
	Preincubation temperatures		
	37°	50°	61°
None	73	24	2
ABA	74	24	2
KGA	82	63	16
KGA + ABA	90	81	21
Pyridoxal-P	100	66	11
Pyridoxal-P + ABA	106	76	11
Pyridoxal-P + KGA	100	100	63
Pyridoxal-P + KGA + ABA	101	92	63

* Activity without preincubation = 100.

all at approximately 1×10^{-3} M concentration, did not affect enzyme activity. Thus, in the isolated enzyme preparation, the reaction rate of ABA-T appeared to be independent of the subsequent enzymatic[4] (22) or nonenzymatic (23) oxidation reactions which can convert succinic semialdehyde to succinate.

[4] R. W. Albers, unpublished data.

A number of substances with pharmacological action in the central nervous system were tested on the ABA-T system. These included strychnine (5×10^{-4} M), procaine (5×10^{-3} M), serotonin (1×10^{-3} M), reserpine (3×10^{-4} M), chlorpromazine (1×10^{-3} M), chloral hydrate (5×10^{-4} M), benactyzine hydrochloride (1×10^{-3} M), sodium diphenylhydantoin (Dilantin 1×10^{-3} M), acetazolamide (Diamox 1×10^{-3} M) and iproniazide (1×10^{-3} M). All were without effect in concentrations far exceeding pharmacologically effective concentrations. Dilantin and Diamox have been reported to elevate ABA levels *in vivo* in the cortex of adrenalectomized rats (24). The enzyme studies suggest that these elevations were not the result of a direct inhibition of ABA-T by these substances.

Substrate Specificity—KGA, pyruvate, oxalacetate and α-ketobutyrate were incubated with ABA and enzyme in the standard system at 37°, pH 8.2, and substrate concentrations of 2.6×10^{-2} M. Of these keto compounds, only KGA was an effective amino group acceptor. This finding is similar to that reported for the ABA-T of *Aspergillus fumigatus* (25). Specificity with regard to amino group donors is less clear-cut. Several ω-amino acids other than ABA transaminated with KGA in the experimental system. Their relative activities are shown in Table IV.

Stabilization of Enzyme Solutions by KGA and Pyridoxal Phosphate—Marked losses of activity were observed when solutions of ABA-T were stored overnight at 4°. Quickly frozen solutions retained activity only if thawed over a period of several hours. A significant degree of inactivation of ABA-T resulted from preincubation of enzyme solutions without substrates or pyridoxal-P at pH 8.2 for 3 minutes at 37°. Preincubation at 50° for 3 minutes resulted in a loss of 70 per cent of the activity and at 61° in virtually complete inactivation (Table V). Either pyridoxal-P or KGA alone, but not ABA, protected against heat inactivation at 50°. At temperatures above 50° the simultaneous presence of KGA and pyridoxal-P afforded more effective protection than either substance alone. ABA did not add appreciably to the protecting effect of pyridoxal-P alone. These results are shown in Table V. The specificity of KGA both as a substrate and as a protecting agent against heat inactivation is noteworthy.

DISCUSSION

ABA (or a metabolite thereof) inhibits stretch receptor and heart preparations of the crayfish (28, 29) and may be the factor which is liberated from inhibitory fibers of the crustacean nervous system (28). Although there is no agreement on a specific mechanism that would explain the role of ABA in the central nervous system of mammals, there is evidence that alterations in ABA levels are accompanied by changes in the electrical activity of the brain (30–35). Vitamin B_6 is probably important in the regulation of ABA levels in the central nervous system (7). Both glutamic acid decarboxylase (the enzyme responsible for ABA synthesis) and ABA-T require pyridoxal-P as cofactor, but the affinity of apoenzyme for coenzyme appears to differ for the two enzymes. Dietary restriction of vitamin B_6 and intraperitoneal injection of semicarbazide have been shown to depress glutamic acid decarboxylase activity *in vivo* (36, 37). When vitamin B_6 deficiency was induced in rats by the administration of convulsant hydrazides (37) or 2-methyl-3-hydroxy-4-methoxymethyl-5-hydroxymethylpyridine hydrochloride (methoxypyridoxine), seizures resulted and 30 to 40 per cent reductions in

cerebral levels of ABA were observed.[5] These results would suggest that of the two enzymes, ABA-T has a higher affinity for the coenzyme. Of interest in this regard is that during the preparation of brain acetone powders, pyridoxal-P was dissociated from glutamic acid decarboxylase to a much greater extent than from the ABA-T.

A comparison of the ABA-T from beef brain and from acetate-adapted *E. coli* revealed significant differences. In contrast to the beef brain enzyme, ABA-T from *E. coli* was not dissociated in acetone powder preparations or activated by pyridoxal-P and was completely and irreversibly inhibited by hydroxylamine.[6] Thus, under the conditions of testing, it appeared as if enzyme molecules with a tightly bound cofactor were irreversibly inhibited by the interaction of hydroxylamine with the aldehyde group of the pyridoxal-P, whereas those with a less tightly bound coenzyme could be reactivated by pyridoxal-P.

SUMMARY

1. A copper chelation method to measure glutamic acid formation by the enzyme γ-aminobutyric acid-α-ketoglutaric acid transaminase from beef brain has been described.

2. The pH-optimum effect of substrate concentration, substrate specificity, temperature effects, time course, and effect of enzyme concentration were investigated.

3. The enzyme was shown to require pyridoxal phosphate for activity and was inhibited by sulfhydryl reagents.

4. The transaminase was not inhibited by anaerobiosis or by a variety of pharmacologically active agents.

5. The heat inactivation of the transaminase was studied and protective effects of α-ketoglutarate and pyridoxal phosphate on enzymatic activity were demonstrated.

Acknowledgments—The assistance of Mrs. W. D. Rowlette in the performance of many of these experiments is gratefully acknowledged.

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N-Terminal Amino Acid Analysis of Growth Hormones from Human, Monkey, Whale, and Beef Pituitary Glands*

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(Received for publication, July 28, 1958)

The isolation of growth hormone (somatotropin) from human, monkey, and whale pituitary glands has recently been reported (1–3). Preliminary studies on the hormone proteins from these species have suggested that phenylalanine is their sole *N*-terminal amino acid residue (1–3), in contrast to the two *N*-terminal residues, alanine and phenylalanine, which have been reported for the bovine somatotropin (4, 5). In this paper are reported the results of an investigation designed to confirm the findings of earlier *N*-terminal studies on human, monkey (rhesus, *Macaca mulatta*), and whale (humpback) growth hormones, and to elucidate a partial *N*-terminal amino acid sequence for the somatotropins from these three species and for the bovine hormone as well.

EXPERIMENTAL

The growth hormone preparations used in this investigation were isolated from the pituitary glands of the various species by methods previously described (1, 3, 6). Oxidized whale growth hormone was obtained by treatment with performic acid according to the procedure of Hirs (7).

The *N*-terminal amino acid residue was determined by the FDB[1] procedure (8, 9). In 400 μl. of 5 per cent $NaHCO_3$, 9.0 mg. of growth hormone were dissolved. To this was added 800 μl. of 2 per cent FDB in ethanol. The reaction mixture was agitated vigorously on a mechanical shaker for 2 hours. The reaction was terminated and the mixture was immediately acidified with several drops of concentrated HCl. The DNP-protein was centrifuged and washed successively with water, ethanol, and ether. After the final washing with ether, dinitrophenol was removed by sublimation in a vacuum overnight. The DNP-protein was then dissolved in 0.5 ml. of constant boiling HCl and, after 16 hours of hydrolysis at 100° the hydrolysate was made 1 N with respect to HCl. Extraction with ether was then performed until no yellow color appeared in the extracts. The ether extracts were then submitted to two-dimensional paper chromatography according to the procedure of Levy (9).

In order to confirm the findings obtained by the FDB procedure as well as to establish the *N*-terminal amino acid sequences in the hormone proteins, the paper strip modification (10) of the phenylthiocarbamyl method of Edman (11) was adopted. To a strip (2 × 8 cm.) of Whatman No. 1 filter paper 0.2 μmole of protein in 50 to 100 μl. of 3 per cent NH_4OH was applied. The paper was dried and treated with 50 μl. of 20 per cent phenylisothiocyanate (in dioxane) and incubated for 12 hours in an atmosphere of pyridine, dioxane, and water at 40°. The strip was washed by being shaken for 15 minutes with five 15-ml. portions of a 1:1 ethanol-ether mixture. The paper strip was then placed in a desiccator which contained beakers of glacial acetic acid and 6 N HCl, and the desiccator was evacuated. After an interval of 5 or 6 hours, the amino acid phenylthiohydantoins split from the protein were washed from the paper by being shaken with 12 ml. of the ethanol-ether mixture for 2 hours. Before the next step, the paper strip was exposed to ammonia fumes to neutralize any final traces of acid which remained from the cyclization step. The ethanol-ether extract, which contained the phenylthiohydantoin, was evaporated to dryness, and the derivative was then identified by the paper chromatographic procedure of Edman and Sjöquist (12). Their procedure was followed exactly, with the "D" (xylene-formamide) and "F" (heptane-ethylene chloride-formic acid) systems. The chromatograms were examined with a fluorescent screen[2] under ultraviolet light and then by the iodide-azide spray (13).

For the investigation of the *N*-terminal amino acid sequence in the bovine hormone, partial acid hydrolysis of bovine DNP-growth hormone was performed and the resulting DNP-peptides were submitted to chromatography on silicic acid-Celite columns (14).[3] In a typical experiment 50 mg. of bovine growth hormone were allowed to react with FDB, the latter in a proportion of 6.5 per cent FDB and 2 per cent $NaHCO_3$ in 66 per cent ethanol; the reaction was allowed to proceed for 2 hours at 25°, with vigorous stirring. The DNP-protein was dried over P_2O_5 after being washed with water, ethanol, and ether. It was then subjected to hydrolysis in concentrated HCl for 4 days at 37° in a sealed tube. The hydrolysate was diluted until it was 3 N with respect to HCl. It was then extracted with 2 × 25-ml. and 5 × 10-ml. portions of ethyl acetate. The pooled

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[1] The abbreviations used are: FDB, fluorodinitrobenzene; and DNP, dinitrophenyl.

[2] Obtainable from the Vogel Luminescence Corporation, San Francisco, California.

[3] We should like to express our gratitude to Dr. W. A. Schroeder of the Gates and Crellin Laboratories of the California Institute of Technology for courtesy extended during a visit by one of us (A. J. P.) to his laboratory, where most of the chromatography of the DNP-peptides was performed.

ethyl acetate extracts were then washed with 10 × 10-ml. portions of water, 2 drops of 6 N HCl being added to 100 ml. of the washing solution. The ethyl acetate extracts were then evaporated to dryness under a vacuum at 37°. The sample was dissolved in 2 ml. of 15AA-5A-B.[4] A No. 1 column (9 × 150 mm.) was prepared as outlined by Green and Kay (14). The column was packed with a 2:1 mixture of silicic acid (Merck, lot No. 40446) and Celite 545 (Johns-Manville) and was prewashed with 0.2 V ml.[5] of ether, 1.5 V ml. of ether-acetone (1:1), 0.8 V ml. of ether, 1 V ml. of ligroin, and finally, 1.1 V ml. of developer. The sample was applied and developer was added. Fast-moving zones were collected in round-bottomed flasks; zones remaining on the column after development were cut from the extruded column of adsorbent and eluted with 25 ml. of a mixture of ethanol and ether in a proportion of 1:4. In the initial stages of fractionation, each zone was submitted to chromatography again in a different solvent system. Usually the initial chromatography was performed with 8AA-4A-L and then 2AA-10A-L or 1AA-5A-B. If zones were slow-moving, a stronger developer such as 3AA-15A-L or 12AA-6A-L was used. The DNP-peptides were hydrolyzed for 16 hours under the same conditions that have been used for determinations of *N*-terminal residues of proteins by the FDB method. The *N*-terminal DNP-amino acid was then submitted to chromatography in two dimensions (9). The aqueous residue which remained after hydrolysis was submitted to chromatography on paper in the system of *n*-butanol-acetic acid-water, 4:1:5, (15) to detect the remaining amino acids in the peptide.

RESULTS AND DISCUSSION

Table I presents the results of *N*-terminal amino acid analysis of growth hormones from various species, performed by the FDB method. It should be noted that the data obtained with the bovine hormone confirm previous findings (4), and that human, simian, and whale somatotropins were found to possess only one *N*-terminal residue, phenylalanine, in an amount of approximately 1 mole per mole of the hormone protein. Examination of the whale growth hormone after oxidation with performic acid again disclosed phenylalanine as the sole *N*-terminal residue; failure to detect cysteic acid indicated the absence of cystine from the *N*-terminus of the native protein. In the oxidized hormone also, the yield of phenylalanine was nearly 1 mole per mole of the hormone protein.

The sole *N*-terminal residue disclosed by the phenylthiocarbamyl procedure for growth hormones from whale, monkey, and human glands was again phenylalanine (Table II). This method confirmed the absence of glycine, proline, and trypotphan, which are subject to poor recovery in the form of their DNP-derivatives during the acid hydrolysis of DNP-protein. It is therefore concluded that these growth hormones possess a single phenylalanine residue at their *N*-terminus. These results, together with the results of the *C*-terminal amino acid determination presented elsewhere (16), strongly support the hypothesis that these growth hormones (whale, monkey, and human) are single-chain polypeptides.[6] Recently, Heijkenskjöld (19) formulated a similar conclusion with respect to the human somatotropin after an investigation of the *N*-terminus of the hormone by the phenylthiocarbamyl procedure.

Stepwise degradation by the Edman method (10, 11) of the growth hormones from human pituitary glands was then carried out. A unique *N*-terminal sequence, Phe.Ser.Thr..., was repeatedly obtained for the hormone from this species (Table II); however, when the whale and monkey somatotropins were investigated by this procedure, certain difficulties were en-

TABLE I

N-terminal residues in various somatotropins as determined by DNP method

Species	Sample No.	Amount used	Moles of *N*-terminal residue* /mole of somatotropin†	
			Phenylalanine	Alanine
		mg.		
Bovine	1	46	0.81	0.96
	2	46	0.94	0.77
Cetacean	1	40	1.15	0
	2‡	40	1.11	0
Simian	1	25	0.60	0
	2	36	0.87	0
Human	1	32	0.82	0
	2	25	0.84	0

* Corrections for destruction of DNP-phenylalanine and DNP-alanine in DNP-somatotropins by acid hydrolysis are taken from earlier studies (4).

† Molecular weights of various somatotropins were assumed as follows (1–3): bovine, 46,000; cetacean, 40,000; simian, 25,000; and human, 27,000.

‡ Performic acid-oxidized sample.

TABLE II

Stepwise degradation of somatotropins from N-terminal sequence by phenylthiocarbamyl procedure

Species	Sample No.	Amino acid identified			Proposed *N*-terminal sequence
		Step 1	Step 2	Step 3	
Bovine	1	Ala, Phe	Phe, Thr	Ala	Ala.(Phe).Ala...
	2	Ala, Phe	Phe, Thr	Ala	Phe.(Thr).Ala...
Human	1	Phe	Ser		Phe.Ser.Thr...
	2	Phe	Ser	Thr	
	3	Phe	Ser	Thr	
Cetacean	1	Phe			
	2	Phe			
	3	Phe			
Simian	1	Phe			
	2	Phe			

[4] The abbreviations of Green and Kay (14) are used to denote the composition of the developer. 15AA-5A-B signifies 15 ml. of acetic acid and 5 ml. of acetone with sufficient benzene to make 100 ml. Similarly, 8AA-4A-L means 8 ml. of acetic acid and 4 ml. of acetone with sufficient ligroin to make 100 ml.; 2AA-10A-L represents 2 ml. of acetic acid and 10 ml. of acetone with ligroin in a volume to make 100 ml.; and 1AA-5A-B is 1 ml. of acetic acid, 5 ml. of acetone, and 94 ml. of benzene.

[5] V refers to the volume of liquid required to moisten completely a column of adsorbent. With a 9 × 150-mm. column this is approximately 7 ml.

[6] These findings do not exclude the possibility of an alkyl group masking the *N*-terminal residue of a second peptide chain in these growth hormones. The presence of acetyl or similar moieties has been shown in tobacco mosaic virus protein (17) and in one of the melanotropins (18).

TABLE III

Some peptides isolated from partial acid hydrolysates of bovine DNP-somatotropin

Peptide No.	*N*-terminal amino acid	Remaining amino acids	Proposed structure
4P8-2	Phe	Ala, Thr	Phe.(Ala, Thr)
4P9-2-1	Ala	Ala, Phe	Ala.(Ala, Phe)
4P9-2-2	Ala	Phe	Ala.Phe.
4P71-1-1	Ala	Phe	Ala.Phe.
4P55-2-2	Phe	Ala, Thr	Phe.(Ala, Thr)

countered at the second step. Because of lack of material, no further studies were conducted on the hormones from these two species.[7]

Fractionation of the DNP-peptides obtained from partial acid hydrolysis of dinitrophenylated bovine somatotropin by the silicic acid-Celite column (14) resulted in DNP-peptides which had either alanine or phenylalanine as their *N*-terminal residue. From these peptides, listed in Table III, it was possible to establish unequivocally the sequence Ala.Phe.Ala... as an *N*-terminal sequence in one of the polypeptide chains of the bovine hormone. It was more difficult to elucidate the sequence in the phenylalanyl chain, for although the tripeptide Phe.(Ala, Thr) was obtained the most frequently, no dipeptide containing either alanine or threonine along with the *N*-terminal phenylalanine appeared. However, application of the stepwise degradation procedure (10, 11) to the native bovine hormone yielded threonine in the second step, together with phenylalanine from the alanyl chain (Table II). It would therefore seem that the sequence Phe.Thr.Ala... is derived from the phenylalanyl chain of beef growth hormone. This and the peptide sequence Ala.Phe.Ala... are proposed as the *N*-terminal amino acid sequences of beef growth hormone.

It is of interest to note that phenylalanine is found as an *N*-terminal residue in all of the growth hormone studied to date. Although the bovine and ovine growth hormones have two *N*-terminal residues (Phe and Ala) (4, 5, 21), the other three hormones seem to consist of only a single chain at the *N*-terminus. This suggests the observation that cattle and sheep are closely related with respect to zoological species, and hence it is not surprising that the hormones derived from these two species should be similar. In the same way man and the monkey are closely related. It is of particular interest in the present findings to note that the whale has been found to be closer to the primates than to the ruminant ungulates.

[7] For a detailed discussion of these difficulties and some proposed *N*-terminal sequences for these two species see (20).

SUMMARY

By means of stepwise degradation by the phenylthiocarbamyl procedure, together with partial acid hydrolysis in the case of bovine growth hormone, it has been possible to demonstrate the following *N*-terminal amino acid residues and adjacent sequences for the growth hormone from several species: beef, alanyl.phenylalanyl.alanyl... and phenylalanyl.threonyl.alanyl...; whale (humpback), phenylalanyl...; monkey (rhesus, *Macaca mulatta*), phenylalanyl...; and human, phenylalanyl.seryl.threonyl... The *N*-terminal residues in these growth hormones have also been established by the fluorodinitrobenzene method.

Acknowledgments—The authors wish to thank Dr. H. Papkoff for many helpful discussions and to acknowledge the generosity of the American Cancer Society and the Albert and Mary Lasker Foundation for research grants.

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The *C*-Terminal Amino Acid Sequence of Growth Hormones from Human, Monkey, Whale, and Sheep Pituitary Glands*

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(Received for publication, July 28, 1958)

In another communication we have reported partial *N*-terminal amino acid sequences for growth hormones (somatotropins) of several species (1); these studies have indicated that whale, monkey, and human growth hormones are single-chain polypeptides at their *N*-terminus. To seek further evidence that these proteins are made up of single polypeptide chains, as well as to make additional comparisons with beef and sheep growth hormones, investigations at the *C*-terminus of these proteins have been carried out by enzymic and chemical means. Results of these investigations are reported herein.

EXPERIMENTAL

Sheep, whale (humpback), monkey (rhesus, *Macaca mulatta*), and human growth hormones were isolated from pituitary glands by previously published procedures (2–5). No evidence of inhomogeneity was observed when these protein preparations were submitted to studies of purity by various physicochemical techniques (3–6). The growth-promoting activity of the hormone was estimated by the tibia test (7) in hypophysectomized rats.

The crystalline carboxypeptidase was a commercial preparation from Worthington Biochemical Corporation. Enzymic digestion was carried out as follows. Growth hormone preparations were incubated with carboxypeptidase in an enzyme to substrate molar ratio of 1:25; the volume was 2 ml. in 1 per cent $NaHCO_3$ with the concentration of substrate 0.4 μmole. The enzyme was pretreated with diisopropyl fluorophosphate to ensure the complete absence of endopeptidase (8). Digestion was carried out at 25°. Aliquots were taken at intervals with a 500 μl. micropipette and transferred to a 10 × 75-mm. culture tube containing 800 μl. of 2 per cent fluorodinitrobenzine in ethanol. The reaction was allowed to proceed for 2 hours with constant shaking on a mechanical shaker. The reaction mixture was then diluted to 6 ml. and excess fluorodinitrobenzene was extracted with three 5-ml. portions of ether. The aqueous solution was then acidified with several drops of concentrated HCl and extraction with 5-ml. portions of ether was again performed until there was no yellow color in the extracts. The pooled ether extracts were evaporated to dryness and submitted to two-dimensional paper chromatography according to the procedure of Levy (9).

The procedure of Niu and Fraenkel-Conrat (10) for the hydrazinolysis (11) of proteins has been employed exactly as these authors have described it, with the exception that an additional extraction of the alkaline reaction mixture with ethyl acetate was performed to ensure removal of 2,4-dinitrophenylhydrazides.

RESULTS AND DISCUSSION

It can be seen in Table I that the growth hormones under investigation all seem to possess *C*-terminal phenylalanine to the extent of 1 mole per mole of protein. A study of the rate of liberation of the various amino acids by carboxypeptidase yields some information about the *C*-terminal amino acid sequences of somatotropin obtained from the various species. With the ovine hormone, the amino acid that is cleaved the most rapidly by the enzyme after phenylalanine is leucine, and then alanine and serine are released in that order (Fig. 1). Hence, the *C*-terminal amino acid sequence of ... Ala.Leu.Phe is proposed for ovine growth hormone. When whale growth hormone is subjected to digestion, a different sequence is indicated, since the alanine was found to be liberated at almost the same rate as the phenylalanine (Fig. 2). With a different sample of hormone from the same species, alanine and leucine were found to be liberated in almost equal amounts, a finding duplicated with a performic acid-oxidized preparation.[1] The data, however, do indicate that alanine is being liberated by carboxypeptidase at a faster rate than leucine. Hence the sequence ... Leu.Ala.Phe is proposed for the *C*-terminus of whale growth hormone.[2] Similarly, from the data in Figs. 3 and 4 the *C*-terminal amino acid sequences ... (Ala,Gly).Phe and ... Leu.Phe may be postulated for monkey and human growth hormones, respectively.

When hydrazinolysis (10, 11) was used to verify the findings derived from the experiments with carboxypeptidase to the effect that phenylalanine is the sole *C*-terminal residue in growth

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[1] The whale hormone was oxidized by performic acid according to the procedure previously described (12). No cysteic acid was liberated from the oxidized hormone by the action of carboxypeptidase. If cysteic acid has become the *C*-terminal residue after oxidation with performic acid, it will be cleaved by carboxypeptidase, as has been demonstrated by experiments with performic acid-oxidized prolactin (13).

[2] A similar sequence has been observed in the *C*-terminus of bovine growth hormone (14).

TABLE I

Quantitative estimation of amino acids liberated by action of carboxypeptidase on somatotropin

Species	Sample No.	Amount used	Time of digestion*	Amino acid liberated				Liberation of phenylalanine†
				Phe	Ala	Leu	Gly	
		mg.	*hrs.*	*μmole*	*μmole*	*μmole*	*μmole*	*moles/mole somatotropin*
Ovine	1	4.6	6	0.097	0.023	0.032	0	0.99
	2	4.6	6	0.093	0.023	0.044	0	0.95
Cetacean	1	4.2	5	0.139	0.072	0.060	0	1.32
	2	4.1	6	0.115	0.102	0.035	0	1.10
Simian	1	2.5	4	0.090	0.025	0	0.036	0.90
Human	1	2.7	6	0.094	0	0.026	0	0.94
	2	3.0	8	0.075	0	0.030	0	0.68

* Enzyme-substrate molar ratio of 1:25; incubated at pH 8.3 and 25°.

† Assumed molecular weights of various somatotropins were as follows (3-6): ovine, 47,000; cetacean, 40,000; simian, 25,000; human 27,000.

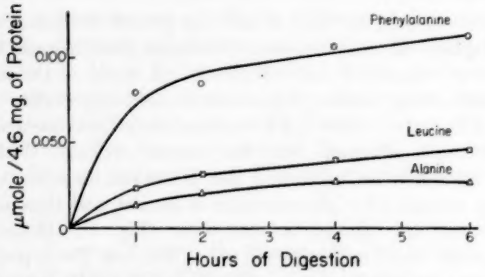

FIG. 1. Rate of release of amino acids by the action of carboxypeptidase on ovine somatotropin. Enzyme-substrate, 1:25; pH 8.4, at 25°.

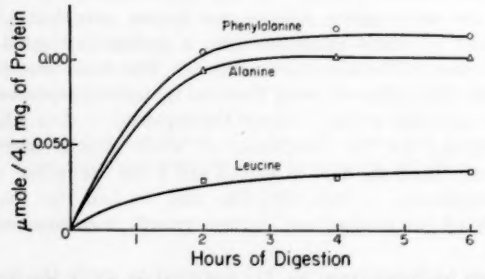

FIG. 2. Rate of release of amino acids by the action of carboxypeptidase on whale somatotropin. Enzyme-substrate, 1:25; pH 8.4, at 25°.

hormone preparations, no amino acids resistant to the action of the enzyme (*e.g.* lysine, arginine, or proline) were detected. As might be expected, phenylalanine was found to be the predominant amino acid released by hydrazinolysis, although traces of other amino acids were observed. The value calculated for the recovery of phenylalanine was corrected for destruction by adding a known amount of phenylalanine to a sample of ovine growth hormone before hydrazinolysis. Another known quantity of phenylalanine was subjected to hydrazinolysis without any protein. When the losses from destruction in the presence and absence of protein were multiplied together, a recovery factor of 2.92 was obtained. Accordingly, the corrected yield of the *C*-terminal phenylalanine residue in the ovine hormone is 0.96 mole per mole of protein, and in the human growth hormone, 0.8 mole.[3] These findings, in conjunction with the results of the carboxypeptidase experiments, clearly show that there is only 1 *C*-terminal phenylalanine residue in ovine and human growth hormones.

[3] It may be recalled that a value of 0.9 mole for *C*-terminal phenylalanine was obtained by earlier hydrazinolysis of beef growth hormone (15).

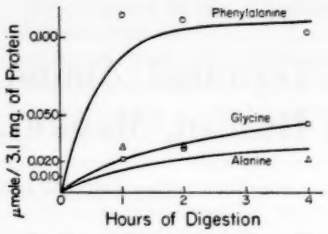

FIG. 3. Rate of release of amino acids by the action of carboxypeptidase on monkey somatotropin. Enzyme-substrate, 1:25; pH 8.4, at 25°.

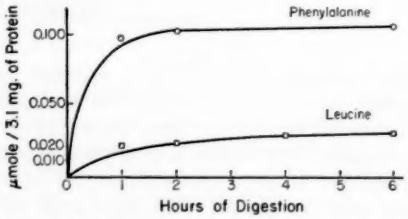

FIG. 4. Rate of release of amino acids by the action of carboxypeptidase on human somatotropin. Enzyme-substrate, 1:25; pH 8.4, at 25°.

TABLE II

Growth-promoting activity of carboxypeptidase-treated somatotropin

Species	Time of digestion*	Tibia test†	
		No. of rats	Width of uncalcified tibial cartilage
	hrs.		*μ*
Ovine	0	6	218 ± 3‡
	12	6	214 ± 3
Cetacean	0	10	237 ± 12
	5	5	242 ± 6
Simian	0	6	246 ± 2
	6	4	242 ± 2
Human	0	6	236 ± 3
	6	10	248 ± 2

* Enzyme-substrate molar ratio of 1:25; incubated at pH 8.3 and 25°.

† Animals given a total dose of 0.04 mg. in 4 days.

‡ Mean ± standard error.

When somatotropins from which 1 residue of phenylalanine had been removed by the action of carboxypeptidase were assayed for growth-promoting activity by the tibia test (7), it was found that *C*-terminal phenylalanine is not essential for the biological function of growth hormones (Table II). These observations are consistent with earlier studies with the bovine hormone (14).

SUMMARY

By means of a combination of two methods, enzymic digestion with carboxypeptidase, and hydrazinolysis, it has been possible to deduce the following *C*-terminal amino acid sequences for growth hormones from several species: sheep, ... Ala.Leu.Phe; whale (humpback), ... Leu.Ala.Phe; monkey (rhesus, *Macaca mulatta*), ... (Ala,Gly).Phe; human, ... Leu.Phe. Moreover, it can be shown that the *C*-terminal phenylalanine residues of these growth hormones are not essential for their biological activity in terms of assay by the tibia test.

Acknowledgments—The authors wish to acknowledge the generosity of the American Cancer Society, and the Albert and Mary Lasker Foundation for research grants.

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The Enzymatic Conversion of 5-Dehydroshikimic Acid to Protocatechuic Acid*

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(Received for publication, March 6, 1958)

Previous studies of the physiology of an aromatic-deficient mutant strain of *Neurospora crassa*, Y7655a (1, 2), have led to the following conclusions: (*a*) The biosynthesis of the aromatic rings of phenylalanine, tyrosine, tryptophan and *p*-aminobenzoic acid proceeds in *Neurospora* as it does in *Escherichia coli* (3) via 5-dehydroshikimic acid and shikimic acid. (*b*) Protocatechuic acid, the major compound secreted by the mutant strain during growth, is derived from DHS[1] (1). (*c*) The observed distributions of carbon atoms 1 and 6 of glucose into PCA was compatible with the notion that the aromatic ring structure was derived by the condensation of a 3- and a 4-carbon fragment derived from glucose (2). (*d*) A comparison of the results obtained for the distribution of carbon atoms 1 and 6 of glucose into shikimic acid accumulated by a mutant strain of *E. coli* (4) with the distribution obtained in PCA accumulated by *Neurospora* led to the assumption that the conversion of DHS to PCA in *Neurospora* involved the loss of the carbonyl oxygen atom at position 5 of DHS (2).

This report primarily concerns the enzymatic conversion of DHS to PCA. Evidence is presented indicating that this conversion does not involve the loss of the carbonyl oxygen atom at position 5 of DHS, but instead is a result of a dehydration in which the hydroxyl oxygen atom at position 3 is lost. Some properties of the enzyme responsible for this conversion, dehydroshikimic dehydrase, are described.

MATERIALS AND METHODS

Biological—The wild type strain of *Neurospora crassa*, SY7A, was used as the source of dehydroshikimic reductase. Strain Y7655-24-24a, an inbred derivative of the original aromatic-deficient mutant Y7655a, was the source of dehydroshikimic dehydrase as well as the source of the enzymes that convert PCA to β-ketoadipic acid. Both strains were usually grown on a minimal medium supplemented with the aromatic amino acids and *p*-aminobenzoic acid as previously described (1).

Pseudomonas fluorescens strain A.3.12, grown on *p*-hydroxybenzoic acid (5), was the source of the bacterial enzymes which convert PCA to β-ketoadipic acid.

Chemicals—DHS was isolated from culture filtrates of the aromatic-deficient mutant 83-2 of *E. coli* strain W, as described by Salamon and Davis (6). The PCA was a commercial product (Reheis Chemical Company) recrystallized twice from water before use. β-Ketoadipic acid was obtained from the Sigma Chemical Company and was recrystallized twice from a petroleum ether-ethyl acetate mixture before use. Shikimic acid was obtained from the California Foundation for Biochemical Research.

Preparation of Extracts—Mycelial mats obtained after 4 days growth at 30° in 2.5-l. Fernbach flasks containing 400 ml. of medium were filtered and washed three times with distilled water. The mycelia were then ground with mortar and pestle for 5 minutes in the presence of washed and ignited sand weighing about one-half the wet weight of the mycelia. After the addition of about 1 ml. of buffer per gm. wet weight of mycelia, the grinding was continued for 5 minutes. The slurry produced was diluted with buffer so that the wet weight to volume ratio was 1:10, and it was homogenized in a Waring Blendor intermittently for 2 minutes at top speed. After an extraction period of from 6 to 10 hours the debris was removed by centrifugation at about 1000 × *g*. All operations subsequent to and including grinding were carried out at 4° to 5°.

Buffers—Best results were obtained when dehydroshikimic reductase was prepared with 0.05 M Tris-HCl buffer at pH 8.0. For the preparation of dehydroshikimic dehydrase, 0.05 M Tris-HCl, pH 7.4, was employed.

Analytical—Estimates of the amount of dehydroshikimic dehydrase activity could be obtained by following the reduction in absorbence at 234 mμ with the use of DHS as substrate ($\epsilon_{234} = 1.1 \times 10^4$, pH 7.2, in 0.05 M Tris-HCl buffer). Since dehydroquinase is usually present in extracts prepared as described, some of the decrease of optical density is the result of the conversion of DHS to 5-dehydroquinic acid, which is essentially transparent in the ultraviolet. However, the equilibrium of this reaction strongly favors DHS (7), and the extensive decrease in optical density observed is largely due to the conversion of DHS to β-ketoadipic acid by dehydroshikimic dehydrase and the PCA-degradative enzymes.

PCA concentrations in reaction mixtures were determined directly by measuring the optical density of the ferric complex of the acid at 640 mμ in a Bausch and Lomb Spectronic 20 with 1-cm. diameter test tube cuvettes. The color of the ferric-PCA complex in buffer is pH-dependent, varying from blue at pH > 7.0 to colorless at pH < 5.0. Assays at pH 5.3 to 5.5 gave consistent extinction coeffecients at 640 mμ. In general, 2 ml. of 0.5 M Tris-maleate buffer, pH 5.2, were added to 3 ml. of reaction mixture (usually buffered at pH 7.4 with 0.05 M Tris-HCl) to bring the pH to 5.3 to 5.4. The optical density

* This investigation was supported in part by Research Grant No. C1267 from the National Cancer Institute of the National Institutes of Health, United States Public Health Service, and by a grant from the American Cancer Society as recommended by the Committee on Growth of the National Research Council.

[1] The abbreviations used are: DHS, 5-dehydroshikimic acid; PCA, protocatechuic acid.

of either 2.9 ml. of the above or of a sample diluted with water was determined after the addition of 0.1 ml. of a 1.0 per cent aqueous $FeCl_3$ solution. The instrument was balanced with a similarly prepared reaction mixture to which no substrate was added. The optical density varies proportionately with PCA concentration between 3 and 20 μg. per ml.

Isotopic—All determinations of radioactivity were made with a gas flow counter and the results expressed as disintegrations per minute corrected to infinite thinness. Samples were counted to within 3 per cent error with suitable corrections for background and coincidence. Wherever desirable, CO_2 was collected and counted as $BaCO_3$.

I am indebted to Dr. D. B. Sprinson for the sample of shikimic acid uniformly labeled with C^{14} in the 3, 4, 5, and 6 positions (8).

EXPERIMENTAL

Manometric—Crude extracts of the aromatic-deficient mutant strain will catalyze the oxidation of DHS with the uptake of 1 mole of O_2 and the evolution of 1 mole of CO_2 per mole of substrate added. The stoichiometry of this reaction is identical to that observed previously for the conversion of PCA to β-ketoadipic acid (9). No O_2 uptake or CO_2 evolution was observed with similarly prepared wild type extracts which have been demonstrated to be deficient in the inducible enzyme, PCA oxidase.

Spectrophotometric—Crude extracts of the aromatic-deficient mutant strain that had been clarified by centrifugation at 30,000 × *g* for 30 minutes catalyzed the complete loss of optical density at 234 mμ when incubated with DHS. However, incubation of DHS with extracts of the wild type strain resulted in a decrease of about 5 to 8 per cent in optical density which could be accounted for by the conversion of DHS to 5-dehydroquinic acid catalyzed by dehydroquinase. This loss of DHS is approximately equivalent to the equilibrium ratio of 15:1, DHS to 5-dehydroquinic acid, reported for the dehydroquinase from *E. coli* at pH 7.4 (7). 5-Dehydroquinic acid has been demonstrated in the equilibrium mixture by the chromatographic and bioautographic procedures described by Davis and Mingoli (10).

No increase in optical density at 290 mμ, the wave length diagnostic for the production of PCA, was observed in reaction mixtures containing extracts of the aromatic-deficient strain and DHS. This was the result, at least in part, of the very high affinity of PCA oxidase for O_2 and PCA, and of the relatively high level of PCA oxidase present in extracts of the mutant. Several attempts to differentially inhibit PCA oxidase were ineffective. *o*-Phenanthroline, an inhibitor of *Neurospora* PCA oxidase (11), did not inhibit the reaction sufficiently at a concentration of 10^{-4} M to allow its use in spectrophotometric experiments. *p*-Chloromercuribenzoate, another inhibitor of PCA oxidase, inhibited the conversion of DHS to PCA.

Enzyme Fractionation—Attempts to separate dehydroshikimic dehydrase and PCA oxidase by conventional ammonium sulfate fractionation coupled with calcium phosphate gel and alumina C γ adsorptions were relatively unsuccessful. Although some separation could be achieved it was never complete and usually resulted in prohibitive losses of dehydroshikimic dehydrase activity. However, a 3-fold increase of the specific activity of dehydroshikimic dehydrase could be obtained by the following method. The crude extract of the aromatic-deficient mutant was adjusted to pH 6.5 with 1 M acetic acid, and protamine sulfate was added until precipitation was complete (about 30 mg. per 100 ml. of extract). The pH was held constant during this operation by the dropwise addition of a 1 M solution of sodium bicarbonate. After removal of the precipitate by centrifugation (10,000 × *g* for 10 minutes), the supernatant solution was brought to 60 per cent saturation with ammonium sulfate. The precipitate obtained was collected by centrifugation (as above) and contained all of the original dehydroshikimic dehydrase activity. The precipitate was then dissolved in 0.05 M Tris-HCl buffer, pH 7.4, and solid ammonium sulfate was added slowly until 32 per cent saturation was obtained. After centrifugation enough ammonium sulfate was added to the supernatant solution to yield 50 per cent saturation. The precipitate that was obtained between 32 and 50 per cent saturation (which usually contained more than 70 per cent of the original activity) was dissolved in 0.05 M Tris-HCl buffer and used immediately whenever possible. All operations were carried out at 4°, since the enzyme is extremely heat labile and more than 40 per cent of the activity is lost within 24 hours at 4°. Storage of preparations at −15° or by lyophilization resulted in excessive losses of activity. Cysteine and/or serum albumin failed to stabilize the preparations. Dialysis against 0.05 M Tris-HCl buffer, pH 7.4, for 12 hours led to excessive losses of activity which were not recoverable by the addition of Mg^{++}, Co^{++}, Fe^{++}, reduced glutathione, cysteine di- and triphosphopyridine nucleotide, flavin mononucleotide and flavin adenine dinucleotide at concentrations between 10^{-6} and 10^{-4} M. The addition of an equal amount of a crude undialyzed extract of the wild type strain also failed to increase activity. The addition of Cu^{++} or Hg^{++} was completely inhibitory.

Procedure for Assay of Enzymatic Activity—Preparations obtained by the procedure described above contained large amounts of PCA oxidase and usually only small amounts of dehydroquinase. In order to prevent the oxidation of the PCA produced from DHS the reaction was run in an O_2-free N_2 atmosphere in 8 ml. modified Thunberg tubes fitted with a double stopcocked head permitting complete flushing with gas. Because of the high affinity of PCA oxidase for O_2, oil-pumped N_2 was passed through alkaline pyrogallol before entering the reaction vessels. Intermittent evacuation of the tubes increased the efficiency of O_2 removal.

The assay mixture usually contained 2.4 ml. of 0.05 M Tris-HCl buffer, pH 7.4, and 0.5 ml. of enzyme preparation containing 10 to 20 units of activity. A unit of activity is defined as that amount of enzyme which catalyzes the conversion of 10^{-9} moles of DHS to PCA per minute under the conditions described. DHS (3 μmoles for maximal initial velocity) in 0.1 ml. of Tris-HCl buffer was added to the side arm and the tubes flushed with N_2 for 1 hour at 0°. After a 10-minute equilibration period at 30° the reaction was initiated by tipping the contents of the side arm into the main compartment. Since more than 90 per cent of enzymatic activity is lost within the 1st minute of incubation at 60°, the reaction was stopped by incubating the reaction vessels for 10 minutes at 60°. 2 ml. of Tris-maleate buffer 0.5 M, pH 5.2, were then added and the reaction mixture heated for an additional 5 minutes at 60°. The flocculent precipitate was removed by centrifugation at 30,000 × *g* for 15 minutes and the supernatant solution assayed for PCA.

Identification of Reaction Product—The absorption spectrum

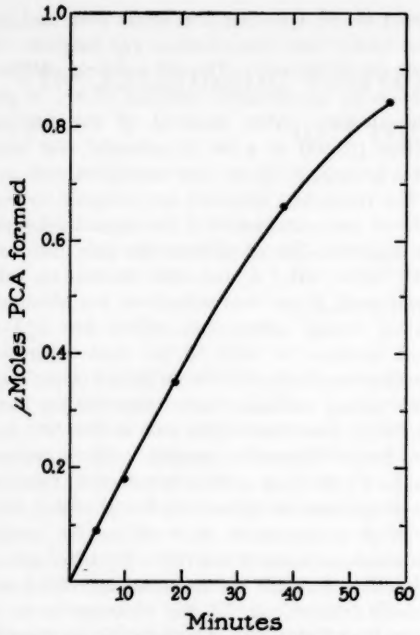

FIG. 1. The kinetics of the conversion of DHS to protocatechuic acid. Each point represents the average of duplicate determinations of protocatechuic acid formed as a function of time. Each flask initially contained 1.78 μmoles of DHS.

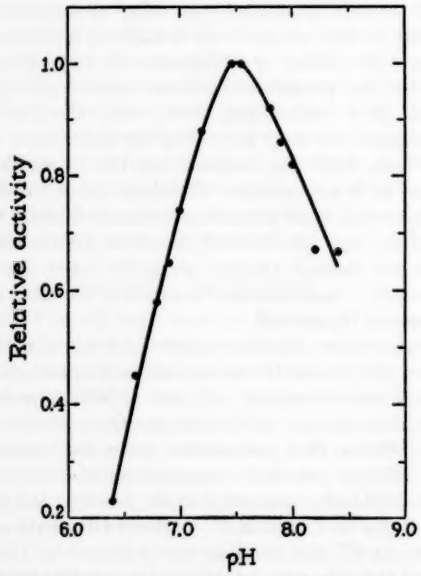

FIG. 2. Relative activity of dehydroshikimic dehydrase as a function of pH. The reaction was carried out as described in the text except that the pH was maintained with 0.05 M Tris-maleate buffer. The reaction was initiated with 3.0 μmoles of DHS and stopped after 15 minutes.

of PCA was identical, between 220 and 300 mμ, with the absorption spectrum of a reaction mixture that had been incubated for 2 hours and corrected for the loss of DHS through its conversion to 5-dehydroquinic acid (about 2 per cent as determined by the decrease in the total optical density at the isosbestic point of DHS and PCA ($\epsilon_{248} = 8.62 \times 10^3$ in 0.05 M Tris-HCl, pH 7.2)), and for residual DHS (approximately 10 per cent). That PCA is the product of the reaction has been amply confirmed by chromatography in butanol-acetic acid (1), the characteristic green $FeCl_3$ color reaction and the isolation of C^{14}-labeled PCA from labeled shikimic acid as described in the isotope experiment below.

Characteristics of Reaction—Although residual DHS is usually observed after prolonged incubation, the reaction is essentially irreversible. It has not been possible to demonstrate the conversion of PCA to DHS when PCA is supplied as substrate. Failure to observe complete conversion of DHS to PCA is probably due to the inactivation of the enzyme on prolonged incubation and the fairly low affinity of the enzyme for DHS.

The kinetics of the conversion of DHS to PCA was followed in order to detect the possible participation of intermediate compounds in the reaction. The data presented in Fig. 1 indicate that there is no significant lag in the production of PCA during the early part of the reaction. This suggests, but certainly does not prove, that the reaction does not involve more than one enzymatic step.

Properties of Enzyme—The relative reaction velocities as a function of pH are plotted in Fig. 2. The pH optimum is between 7.4 and 7.6. The dissociation constant of the enzyme substrate complex is fairly high ($K_m = 6.0 \times 10^{-4}$ M). The enzyme is completely inhibited by *p*-chloromercuribenzoate (10^{-4} M). This inhibition is reversed by reduced glutathione (5×10^{-4} M) added either before or simultaneously with the initiation of the reaction. Addition of reduced glutathione after 10-minute preincubation of the enzyme with *p*-chloromercuribenzoate restored 5 to 10 per cent of the original activity. The enzyme therefore seems to have a thiol group which is necessary for activity.

Isotopic Experiments—The experiments reported above suggest that the conversion of DHS to PCA involves only one enzymatic reaction. The simplest mechanism for the conversion would involve dehydration across positions 2 and 3 of the enol form of DHS. However, the consequent loss of the oxygen atom at position 3 of DHS is incompatible with the previously postulated loss of the carbonyl group at position 5 (2). It was therefore necessary to distinguish rigorously between the two possibilities.

Shikimic acid, uniformly labeled with C^{14} in the 3, 4, 5, and 6 positions, was converted to PCA. This was accomplished by first converting shikimic acid to DHS with dehydroshikimic reductase obtained from the wild type strain of *Neurospora*. The DHS was then converted to PCA with dehydroshikimic dehydrase.

The properties of *Neurospora* dehydroshikimic reductase are quite similar to the analogous enzyme from *E. coli*. Except for the use of protamine sulfate instead of $MnCl_2$ to remove nucleic acids, the *Neurospora* enzyme was purified essentially as described for the enzyme from *E. coli* (12). The TPN-dependent conversion of shikimic acid to DHS was coupled with glutathione reductase as described by Yaniv and Gilvarg (12). After the complete conversion of shikimic acid to DHS, as judged by the production of sulfhydryl groups, the enzyme was heat-inactivated at 60° for 15 minutes. Preparations of dehydroshikimic dehydrase were added to the reaction mixture at 0° after adjusting the pH to 7.4. The reaction vessels were

then incubated at 30° in an N_2 atmosphere until completion of the reaction. Direct coupling of dehydroshikimic reductase with dehydroshikimic dehydrase was not feasible because the dehydroshikimic dehydrase preparations contained excessive TPN-destroying activity. The over-all yield of PCA from shikimic acid was 60 per cent.

The reaction mixtures were adjusted to pH 3 and continuously extracted with ether for 16 hours. PCA was obtained from the ether extract after the addition of carrier and recrystallized to constant activity from a benzene-ethyl acetate mixture.

The operational considerations involved in resolving the primary issue, that is, whether the oxygen atom at the 3 rather than the 5 position is lost during the conversion of DHS to PCA, are illustrated in Fig. 3. If the oxygen atom at position 3 is lost, the PCA obtained (PCA I) would be oriented so that the hydroxyl groups would be those originally attached to carbon atoms 4 and 5 of shikimic acid. If the oxygen atom at position 5 of PCA is lost (PCA II), the hydroxyl groups remaining would correspond to those attached to carbon atoms 3 and 4 of shikimic acid.

The distributions of the C^{14} in PCA I and PCA II can be distinguished by taking advantage of the equal derivation of the carbonyl carbon atom of β-ketoadipic acid from the 1 and 2 position of PCA I (or the 1 and 6 position of PCA II) when PCA is converted to β-ketoadipic acid by extracts of *Pseudomonas fluorescens*. In contrast to the above, the carbonyl carbon atom of β-ketoadipic acid, when produced from PCA by the enzyme system from *Neurospora*, is derived solely from carbon atom 2 of PCA I (or carbon atom 6 of PCA II) (9).

As illustrated in Fig. 4, although two different distributions of radioactivity in the β-ketoadipic acid should be obtained from PCA I and PCA II by the use of the enzyme system from *Neurospora* and from PCA I by use of the enzyme system from *Pseudomonas*, the radioactivity should in each case be equally distributed among 4 of the 6 carbon atoms. In contrast, only the carboxyl carbon atoms of β-ketoadipic acid derived from PCA II by the action of the *Pseudomonas* enzymes would retain the specific activity of the corresponding carbon atoms of PCA, whereas each of the remaining carbon atoms would contain 12.5 per cent of the total radioactivity. Hence a determination of the specific activity of the methylene carbon atom relative to the activity of the α-carboxyl carbon atom of β-ketoadipic acid derived from PCA by the enzymatic activity of extracts of *Pseudomonas* should be sufficient to distinguish between PCA I and II.

The C^{14}-labeled PCA obtained from shikimic acid was subjected to two separate degradations. Part of the PCA was converted to β-ketoadipic acid with extracts of *Pseudomonas* grown on *p*-hydroxybenzoic acid. Each of the samples of β-ketoadipic acid was decarboxylated, and the levulinic acid obtained was converted to iodoform and succinic acid as described previously (2). The succinic acid was isolated from the reaction mixture by continuous extraction with ether and repeatedly sublimed and crystallized from benzene-ethyl acetate until constant activity was obtained. The iodoform derived from the methyl carbon atom of levulinic acid (which corresponds to the methylene carbon atom of β-ketoadipic acid) was oxidized to CO_2 (2).

The data obtained from the isotope experiment are presented in Table I. Within the limits of experimental error the β-ketoadipic acid obtained from PCA by either the *Neurospora* or

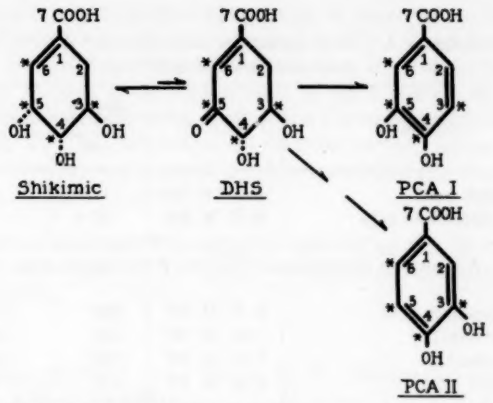

FIG. 3. Possible orientations of protocatechuic acid (PCA) derived from shikimic acid labeled with C^{14} at positions denoted by ✳.

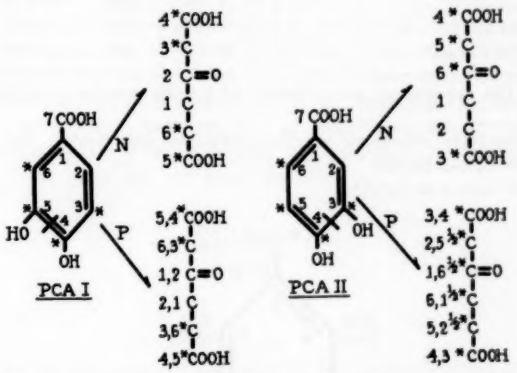

FIG. 4. The distribution of C^{14} in β-ketoadipic acid derived from protocatechuic acid (PCA I and PCA II) with enzyme preparation from *Neurospora* (*N*) and *Pseudomonas* (*P*). Labeled carbon atoms are denoted by ✳.

Pseudomonas enzymatic conversion contained about 50 per cent of the total C^{14} equally distributed between the α-carboxyl and methylene carbon atoms. The remaining C^{14} was found in the succinic acid. Hence, as indicated in Fig. 4, the PCA obtained from 3,4,5,6,C^{14}-shikimic acid must have retained the oxygen atoms at the 4 and 5 positions of shikimic acid. The conversion of DHS to PCA seems therefore to involve the loss of the oxygen atom at position 3 of DHS.

DISCUSSION

The isotopic evidence presented above suggests that DHS most probably is converted to PCA by dehydration across positions 2 and 3 of the enol form of DHS. The loss of the oxygen atom at position 3 of DHS rather than at position 5 necessitates a revised interpretation of the distribution of the 1 and 6 carbon atoms of glucose in PCA presented in an earlier report (2). At that time position 2 of PCA was assigned to the site of high relative incorporation of glucose carbon atom 1, and position 6 was assigned to that of high relative incorporation of glucose carbon atom 6 on the basis of the overt similarity of the distribution of the two glucose carbon atoms into the PCA of *Neurospora* and shikimic acid of *E. coli* (4). It was implicit in

TABLE I

*Distribution of C^{14} in β-ketoadipic acid obtained from 3, 4, 5, 6-labeled shikimic acid**

Compound	Activity†	Dilution relative to shikimic acid	Activity relative to β-ketoadipic acid
Shikimic acid	1.44×10^6		
Protocatechuic acid	8.73×10^3	16.5	
Neurospora conversion of PCA to β-ketoadipic acid			
β-Ketoadipic acid	6.50×10^2	222	1.0
α-Carboxyl‡	1.61×10^2	222	0.25
Methylene‡	1.83×10^2	222	0.28
Succinic acid	3.12×10^2	222	0.48
Pseudomonas conversion of PCA to β-ketoadipic acid			
β-Ketoadipic acid	11.1×10^2	129	1.0
α-Carboxyl‡	2.89×10^2	129	0.26
Methylene‡	3.05×10^2	129	0.27
Succinic acid	5.55×10^2	129	0.49

* The experiment was initiated with 0.091 mmoles of shikimic acid.

† Activity expressed as disintegrations per minute per 0.1 mmole of compound.

‡ Counted as $BaCO_3$.

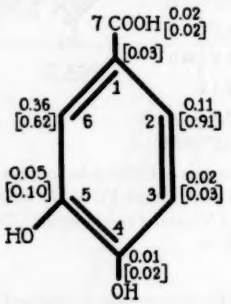

FIG. 5. The distribution of glucose carbon atom 1 and 6 in protocatechuic acid. The values for glucose carbon atom 6 are in brackets. All activities are expressed in terms relative to glucose supplied. The data have been obtained from Tatum and Gross (2) and revised as indicated in the text.

this assumption that the orientation of PCA relative to shikimic acid was such that the hydroxyl groups at positions 3 and 4 of DHS were retained during its conversion to PCA (Fig. 3, PCA II).

The observed loss of the oxygen atom at position 3 of DHS, however, yields a PCA in which the oxygen atoms at positions 4 and 5 of DHS are retained (Fig. 3, PCA I). The oxidation of this PCA by the *Neurospora* enzyme system leads to the production of a β-ketoadipic acid in which the carbonyl carbon atom is exuivalent to that at position 2 of PCA and DHS rather than position 6 as assumed previously (2) (Fig. 4). Therefore, the values of the incorporation of glucose carbon atoms 1 and 6 into positions 2 and 3 of PCA, as published previously (2), really corresponded to the incorporation in the 6 and 5 positions of DHS and shikimic acid; the incorporation into the 6 and 5 positions of PCA corresponded to the incorporation into the 2 and 3 positions of DHS and shikimic acid.

The incorporation of glucose carbon atoms 1 and 6 into PCA obtained previously (2) is presented in Fig. 5 as revised in the light of the above. In this figure the structure of PCA is presented so that the carbon atoms of PCA and the oxygen atoms attached thereto correspond to those of DHS and shikimic acid (as in PCA I, Figs. 3 and 4). The ratio of incorporation of carbon atom 6 to carbon atom 1 of glucose into the 6 position of PCA is now roughly equivalent to the incorporation observed in the 6 position of shikimic acid (1.7 to 1 *versus* 2.4 to 1). However, the 8.3 to 1 ratio of glucose carbon atom 6 to carbon atom 1 incorporated into the 2 position of PCA is quite different from the 1.3 to 1 ratio reported for shikimic acid obtained from *E. coli* (13).

The distribution of glucose carbon atoms in PCA obtained from *Neurospora* is difficult to reconcile with the demonstrated condensation of a 3- and 4-carbon unit derived fairly directly from glucose into a 7-carbon precursor of 5-dehydroquinic acid, DHS, and shikimic acid in *E. coli* (14). If a similar condensation were involved in the corresponding synthesis in *Neurospora*, then the 3-carbon unit would correspond to carbon atoms 7, 1, and 2 of PCA and the 4-carbon unit would correspond to the carbon atoms at positions 3, 4, 5, and 6 (8, 13). The observed preponderance of the incorporation of glucose carbon atom 6 into the 2 position of PCA might result from the loss of carbon atom 1 of glucose as a consequence of extensive oxidation of glucose via 6-phosphogluconate (2) yielding a pool of 3-carbon units containing an excess of glucose carbon atom 6 relative to carbon atom 1. However, a correspondingly large excess of glucose carbon atom 6 relative to carbon atom 1 would be expected in the 4-carbon unit pool. The extensive derivation of the carbon atom at position 6 of PCA from carbon atom 1 of glucose relative to the carbon atom at position 2 is therefore not consistent with expectation.

Although it is possible to reconcile the distribution of glucose carbon atoms in PCA with the distribution reported for shikimic acid from *E. coli* (13) by making certain assumptions about 3- and 4-carbon precursor pools, the data obtained are insufficient for an adequate analysis. An evaluation is rendered more difficult by the fact that PCA is accumulated during the active growth phase of *Neurospora*, whereas shikimic acid is produced almost exclusively after growth of the mutant strain of *E. coli* has ceased.[2] This difference would be expected to affect the labeling pattern, irrespective of the basic metabolic differences between the two organisms.

In any case, the presence in *Neurospora* of the enzymatic apparatus for the synthesis of shikimic acid from 5-dehydroquinic acid and the physiological characteristics of the aromatic-deficient mutant strain (1) indicate that the major terminal synthetic steps in the synthesis of shikimic acid are the same in *E. coli* and *Neurospora*. Further discussions of the isotopic data obtained and the nature of the precursors of 5-dehydroquinic acid must be reserved until more detailed isotopic and enzymatic analyses have been performed.

Dehydroshikimic dehydrase, like PCA-oxidase (9), is present as part of the constitutive enzymatic complement of the aromatic-deficient mutant strain but is normally absent from the

[2] B. D. Davis, personal communication.

wild type strain. Presumably, dehydroshikimic dehydrase is present in the mutant strain because of the accumulation of DHS during growth. Although *Neurospora* synthesizes DHS as a precursor for its aromatic amino acids, it would seem that normally either DHS is not produced in sufficient quantities to induce the synthesis of dehydroshikimic dehydrase or that the DHS synthesized does not reach the enzyme-forming site.

SUMMARY

Some properties of dehydroshikimic dehydrase, the enzyme responsible for the conversion of 5-dehydroshikimic acid to protocatechuic acid, are described. Evidence is presented which indicates that 5-dehydroshikimic acid is converted to protocatechuic acid by dehydration across positions 2 and 3 of the enol form of 5-dehydroshikimic acid; thus this conversion involves the loss of the oxygen atom at the 3 position of 5-dehydroshikimic acid rather than at the 5 position as previously assumed. The distribution of glucose carbon atoms into protocatechuic acid reported previously is revised and discussed in the light of the above.

Acknowledgments—The author is indebted to Drs. B. Davis, D. Sprinson, and E. L. Tatum for helpful discussions of the manuscript.

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Role of the Ribonucleoprotein Particle in Protein Synthesis and the Effects of Growth Hormone*†

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(Received for publication, June 3, 1958)

A relationship between ribonucleic acid and protein synthesis was first suggested by Caspersson (2) and Brachet (3). In mammalian liver the microsomes, which contain most of the cytoplasmic ribonucleic acid, incorporate labeled amino acids very rapidly (4) and are intimately involved in protein synthesis. The nature of the relationship between the protein and nucleic acid of the ribonucleoprotein remains obscure; whether protein synthesis is normally accompanied by nucleic acid renewal is controversial. A possible cause of this ambiguity is the lack of work done on material of known purity.

Most of the microsomal ribonucleic acid is in the form of RNP[1,2] (5, 6). When microsomes from the livers of animals which have received labeled amino acids are separated into RNP and lipoprotein fractions, the protein of the particles is found to be labeled much more rapidly than the microsomal lipoprotein (7). Furthermore, amino acids are incorporated *in vitro* into crude ribonucleoprotein prepared from ascites tumor cells (8). In electron micrographs of thin sections of liver cells these ribonucleoproteins appear as dense granules 100 to 150 A in diameter, usually attached to the endoplasmic reticulum (9); in electron micrographs of separated microsomes these granules are found attached to isolated fragments of reticulum (10).

Highly purified RNP can be isolated from rat liver (6). These particles have been characterized with the analytical ultracentrifuge and electrophoretically (5, 6). Such preparations contained approximately 4 per cent of ferritin, but are free of other proteins. In the metabolic experiments described below the role of RNP in protein synthesis has been investigated with such preparations.

EXPERIMENTAL

Materials—Glycine-1-C^{14} and adenine-8-C^{14} were obtained from the Isotopes Specialties Co., Burbank, California, and L-methionine-S^{35} from the Abbott Laboratories, North Chicago, Illinois. The glycine was diluted with unlabeled glycine to a final activity of 0.81 μc. per μmole. The activity of the adenine was 8.9 μc. per μmole and that of the methionine at the beginning of the experiments was 2.4 μc. per μmole. Part of the growth hormone was a highly purified bovine preparation distributed by the National Institutes of Health and part was obtained from the Armour Laboratories, Kankakee, Illinois.

Incorporation Studies—Male Wistar rats, which weighed between 150 and 200 gm., were anesthetized by the intraperitoneal injection of 45 mg. of sodium phenobarbital per rat. The precursor was then injected into the tail vein, in the following amounts: glycine 0.1 mmole per kg., adenine 0.002 mmole per kg., or methionine 0.0165 mmole per kg. of body weight. The rats were killed at 15, 30, 45, 60, 120, and 1020 minutes after the administration of the isotope. Blood was collected by heart puncture before the rats were killed. The livers were excised and immediately placed on ice and pooled. Seven to nine rats were used in each experiment and at least 60 gm. of liver were obtained.

In the experiments where the effect of growth hormone was studied, each animal was given 410 μg. of hormone in pyrogen-free water intraperitoneally per day for 2 weeks. The treated animals gained about 20 per cent of their weight per week whereas animals injected with pyrogen-free water gained only 12 per cent.

Isolation of RNP and Liver Supernatant Proteins—In each experiment RNP was isolated from the pooled livers. In the earlier experiments up to No. 296, the preparations were carried out as described previously (6), except that the rats were not fasted during the preceding night, and the livers were not perfused. The crude RNP was washed four times by alternate cycles of low- and high-speed centrifugation, with stabilizing factor prepared from calf liver (6).

In the later experiments, the RNP was washed three times, with a buffer containing 5×10^{-4} M K_2HPO_4, 5×10^{-4} M KH_2PO_4, and 5×10^{-4} M $MgCl_2$, at pH 6.8 (11). The three low-speed centrifugations were carried out in the 40 rotor of the Spinco model L ultracentrifuge. The first two were at 12,900 × *g* for 15 minutes, and the last was at 26,000 × *g* for 15 minutes.

The proteins which remained in the supernatant solution after the sedimentation of the crude RNP were precipitated by the addition of two volumes of cold alcohol. This fraction, referred to in the text as the supernatant proteins, includes not only the soluble liver proteins of low molecular weight, but also the microsomal lipoproteins which are soluble in 0.5 per cent sodium deoxycholate.

Electrophoretic Analysis—A solution of RNP in 0.02 M $NaHCO_3$ plus either purified stabilizing factor (6) or the phosphate magnesium buffer was analyzed in a 2-ml. cell. The RNP

* Supported in part by grants from the National Cancer Institute, National Institutes of Health, Public Health Service (Grant No. CY-3190), and from the Atomic Energy Commission (Contract No. AT (30-1)-910). Part of the growth hormone used was a gift from the Endocrinology Study Section, National Institutes of Health.

† This paper has been presented in part (1).

[1] The exact nature of these particles and their relationship to the microsomes are discussed in more detail elsewhere. M. L. Petermann and M. G. Hamilton, to be published.

[2] The abbreviation used is: RNP, ribonucleoprotein particle(s).

concentration was about 10 mg. per ml. A potential gradient of 6.5 volts per cm. was applied for 60 min. The contamination with ferritin was estimated by comparing the photographs with previously analyzed pictures. Whenever the ferritin boundary appeared to be more than 4 per cent of the total area, the picture was traced and the area measured by planimetry.

Ultracentrifugal Analysis—Solutions in 0.1 M $NaHCO_3$ (6) containing about 4 mg. per ml. of RNP were analyzed in the Spinco model E ultracentrifuge.

In the growth hormone experiments difficulty was encountered in washing the nucleoprotein free of turbid impurities. In three experiments, 303, 308, and 314, the turbid material sedimented only part way down the tube in the final low-speed centrifugation. The clear upper layer was used for the electrophoretic and ultracentrifugal analyses, and for the determination of the specific activity of the amino acids in the protein. The lower turbid layer was used only for measuring the activity of the RNA purines.

Determination of Radioactivity—In those experiments where the incorporation of glycine was studied, the RNP was treated with 2,4-dinitrofluorobenzene in ethanol (12). Free amino groups from terminal amino acids in the protein as well as any free glycine present were thus converted into dinitrophenyl derivatives. The treated RNP was then hydrolyzed with 6 N HCl at 120° for 16 hours. The hydrolysate was evaporated to dryness in a vacuum, the residue was dissolved in water, and any dinitrophenyl derivatives were extracted with ether. The aqueous layer was made alkaline (pH 8 to 9) and was again treated with 2,4-dinitrofluorobenzene. The dinitrophenyl derivatives of nonterminal glycine and serine as well as the other nonbasic amino acids were extracted with ether at pH 4 to 6. The aqueous phase was saved for the isolation of the RNA purines. The dinitrophenyl glycine and serine were isolated by chromatography of the ether soluble fraction on a Celite column by the method of Perrone (13) and their identify was confirmed by paper chromatography. The amounts of the glycine and serine derivatives were determined by their absorption, at 330 mμ, in chloroform solutions. A value of 14.3×10^3 was used for the molecular extinction coefficient. Aliquots of the solutions of the dinitrophenyl derivatives were plated on aluminum planchets and the radioactivity was determined. The serine and glycine of the supernatant protein and of the serum proteins were isolated and their radioactivities determined in the same manner.

The adenine and guanine of the RNA were isolated by precipitating the purines as their silver salts from the aqueous solution after the removal of the glycine and serine derivatives. The purines were regenerated with HCl, the adenine and guanine isolated by paper chromatography, and the radioactivity was assayed as infinitely thin films (14) with an internal Geiger-Müller flow counter (Radiation Counter Laboratories, Inc., mark 12, model 1, helium isobutane gas). Triplicate determinations of radioactivity were made and the extremes were within 15 per cent of the means.

Acid-soluble purines were isolated from some of the liver prepared in conjunction with Experiment 291. The tissue was extracted with cold 5 per cent trichloroacetic acid, the barium nucleotides were precipitated, the barium salts hydrolyzed with acid at 100°, and the purines isolated as described above.

In those experiments in which methionine-S^{35} and adenine-8-C^{14} were used as precursors, the RNP was washed with cold 5 per cent trichloroacetic acid which contained, as carriers, 500 μg. per ml. of nonradioactive adenine and methionine. The precipitate was then treated with hot 5 per cent trichloroacetic acid to extract the nucleic acid purines. The purines thus obtained were precipitated as silver salts which were treated in the same manner as described above. The protein residue was washed with alcohol, alcohol-ether (3:1) and ether, and then air dried. Approximately 10 mg. of protein were weighed and dissolved in 1 ml. of 1 N sodium hydroxide. The solution was diluted to 10 ml. and 1 ml. aliquots were plated on 10 $cm.^2$ stainless steel planchets. In order to assure even plating, 0.1 ml. of a 0.02 per cent solution of Triton X-100 (Rohm and Haas Co., Philadelphia) was added. All radioactivity measurements were made with an internal Geiger-Müller flow counter. The self-absorption correction was small (5 to 9 per cent), and could therefore be neglected in the calculations. Aliquots of the protein solutions were analyzed for nitrogen, and the specific activity was calculated as counts per minute per mg. of nitrogen.

RESULTS

Electrophoretic Analyses—The electrophoretic patterns obtained with the 2-ml. cell were similar to those obtained in the standard cell (6). They showed large RNP boundaries, with some turbidity, probably aggregated RNP, traveling with a shoulder on the slow side on the main peak. The ferritin contents ranged from 2 to 9 per cent, with a mean of 4 per cent. Since both the nucleoprotein and the ferritin are about 60 per cent protein (6), apoferritin contributed about 4 per cent of the protein. No microsome protein boundary (6) was seen. Most of the preparations contained large amounts of glycogen, since the rats had not been fasted.

Ultracentrifugal Analyses—Most of the patterns resembled those reported previously. Component A′ accounted for about 1 per cent; A, 11; B, 60; C, 10; and E, 11 per cent of the total RNP (6). In several preparations the proportions of A′ and A were increased to 11 per cent and 29 per cent, respectively.

Metabolic Results—Tables I and III show that, with either protein precursor, the particle protein reached maximum activity in 15 minutes or less, whereas the serum and supernatant

TABLE I

Incorporation of glycine-1-C^{14} into proteins and RNP-purines

Experiment No.	Time	Radioactivity*						
		RNP				Supernatant	Serum	
		A†	G	Gly	Ser	Gly	Gly	Ser
	min.							
276	15	8	3	500		330		
291	15	‡		532				
282	30	14	3	322	279			
283	45	15	4	300		106	330	
286	60	46	6	140		373	228	283
301	60	28	3	250	510	1095		
289	120	24	16	213		977	485	655
294	1020	37	23	243	540	320	1044	788

* Values given are c.p.m. per μmole.

† A, adenine; G, guanine; Gly, glycine; Ser, serine.

‡ Adenine of acid-soluble nucleotides contained 256 c.p.m. per μmole.

TABLE II

Incorporation of glycine-1-C^{14} into proteins and RNP-purines in growth hormone-treated animals

Experiment No.	Time	Radioactivity*							
		RNP				Supernatant		Serum	
		A†	G	Gly	Ser	Gly	Ser	Gly	Ser
	min.								
309	15	18	2	374	855	461	193	11	7
312	30	4	4	159	537	955	704		
303	30	9	1	162	474	965		89	119
314	60	6	1	195	272	2342	290	689	1389
308	1020	42	69	85	29	481	348	531	530

* Values given are c.p.m. per μmole.

† A, adenine; G, guanine; Gly, glycine; Ser, serine.

proteins continued to increase in activity for a considerable length of time. There was relatively little C^{14} in the RNA until 45 minutes after administration of the isotope. The radioactivity of the RNP protein fell to half its maximal value during the course of the experiment, whereas there was no parallel drop in the purines.

It is interesting to note that with either nucleic acid precursor (Tables I and III) the label reached the RNA adenine much more quickly than it reached the RNA guanine. This is not surprising when adenine itself is the precursor, but would not have been expected *a priori* with glycine.

In one 15-minute experiment a larger number of animals was used. Some of the livers were extracted with cold 10 per cent trichloroacetic acid rather than treated for the isolation of RNP. The acid-soluble adenine was very highly labeled.

The supernatant protein reached its maximal radioactivity 1 to 2 hours after administration of the isotopic glycine and more than 45 minutes after the injection of labeled methionine. With both these precursors the specific activity of the supernatant protein fell at such a rate that at 1020 minutes it had about the same value as at 15 minutes. The serum protein may never have reached maximal activity in the experiments with glycine (Table I), but with methionine the activity at approximately 1 hour was essentially as high as it ever became (Table III). The values obtained for serine (Table I) reached their maximum at a later time than did those for glycine.

TABLE III

Incorporation of methionine-S^{35} and adenine-8-C^{14} into proteins and RNP-purines

Experiment No.	Time	Radioactivity*				
		RNP			Supernatant	Serum
		A†	G	Prot.		
	min.					
296	15	2	6	6187	5,222	500
310	30	15	8		5,361	1291
304	45	106	10	4487	12,482	3024
302	60	77	4	3640	6,343	7020
300	120	117	6	4538	7,032	5636
295	1020	72	72	2801	5,089	9700

* The A and G values are c.p.m. per μmole. The protein activities are given as c.p.m. per mg. N, corrected for decay and reported as of the date of the beginning of the experiment.

† A, adenine; G, guanine.

In experiments in which growth hormone was administered, a very definite effect on the rate of synthesis of the protein can be detected (Tables I and II). The time required to reach maximal activity in the serum protein was greatly reduced, as was true also with the RNP and supernatant proteins. Apparently, under the influence of growth hormone the rate of RNP protein synthesis was increased so that the maximal activity was reached in less time than in the control animals. Whether this is the correct interpretation of the data or not, it is obvious that the incorporation of glycine into the particle protein was considerably altered by the administration of growth hormone. By contrast, there was no pronounced effect upon the rate of synthesis of the adenine of the nucleoprotein particle; and the radioactivity of the guanine was increased only in the 17-hour experiment.

The activity of the serine of both the serum and the particles showed a definite response to growth hormone treatment. These data imply that the hormone has an effect on the synthesis of serine which is independent of the stimulation of protein synthesis induced by the hormone.

DISCUSSION

The data presented confirm the oft-repeated observation that injected amino acids appear more quickly in microsomal protein than in supernatant proteins and serum proteins, and further they show that this is true of the RNP protein itself. The variation with time of the radioactivity of the RNP protein and that of the supernatant and serum proteins is not inconsistent with a precursor-product relationship, but it is difficult to say that such a relationship has been established. These findings are similar to those of other workers.[3]

The data presented here indicate that RNP nucleic acid was not appreciably labeled during the first 45 minutes, while the protein reached maximal activity in 15 minutes or less. The delay with glycine as a precursor cannot be attributed to the time required for the synthesis of the purine ring, since the soluble adenine nucleotides were labeled rapidly, and administration of adenine did not result in a more rapid entrance of label into the purines of the nucleic acid. This may indicate that the RNA and the protein of the RNP are not renewed simultaneously. An alternate hypothesis is that there are two protein entities in the RNP, one which is made concurrently with the nucleic acid and has the same turnover, and the other which is more labile. Presumably this labile portion is a protein being synthesized by the particle, whereas the stable portion, in conjunction with RNA, forms the matrix, as suggested by Littlefield *et al.* (7).

The rats treated with growth hormone gained weight more rapidly than the controls. The rate of appearance of label in the three protein fractions was also increased, although the maximum was not observed for the RNP protein since it occurred in less than 15 minutes. The slope of the decay curve was greater in treated than in control rats (the 1-hour value was somewhat less than the control, and the 17-hour value was one-third that of the control). One possible interpretation of these results is that the maximum was greater after growth hormone

[3] This work has been discussed in several review articles (4, 15, 16).

treatment. In the particles the utilization of glycine as a protein precursor was quantitatively changed while at the same time there was no demonstrable effect on the role of glycine as an RNP purine precursor. Since the rate of protein anabolism was changed, with no effect on RNA anabolism, concurrent synthesis of nucleic acid and of all the protein could not have occurred.

It seems, therefore, that if the RNP nucleic acid is involved in protein synthesis, it is not serving as an active template but is performing a more passive function, and that the life of this template is not controlled primarily, if at all, by the rate of protein synthesis.

SUMMARY

The incorporation of labeled glycine, adenine, and methionine into the microsomal ribonucleoprotein particles of rat liver and into supernatant and serum proteins has been determined. The nucleic acid purines reached maximal activity much later than did the protein of the particles. The administration of growth hormone before the administration of labeled precursors resulted in an increased rate of labeling of the proteins, but not in a parallel change in the activities of the purines of the particles. The data suggest that there is not necessarily a simultaneous synthesis of the total protein and nucleic acid of the ribonucleoprotein particle.

A pronounced effect of growth hormone on the synthesis of serine was noted.

Acknowledgments. The authors wish to thank Dr. George B. Brown for his interest in this work and his many helpful suggestions. They are also indebted to Miss B. Keshner and Miss P. Pecora for their help in the performance of the experimental work.

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Isoleucine and Valine Metabolism in *Escherichia coli*

VIII. THE FORMATION OF ACETOLACTATE*

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(Received for publication, June 9, 1958)

On the basis of isotopic results obtained with *Torulopsis utilis* and *Neurospora crassa*, Strassman *et al.* (1) and Adelberg (2) have proposed a mechanism for valine biosynthesis in which pyruvate is converted to α-acetolactate. Acetolactate would then undergo a rearrangement to yield the carbon skeleton of valine. Abelson (3) showed that pyruvate, in competition with glucose, gave rise to the carbons of valine in *Escherichia coli*. This paper will show that extracts of *E. coli* do indeed have an acetolactate-forming enzyme, and evidence will be presented that this enzyme plays an essential role in valine biosynthesis. Preliminary reports of part of this work have been presented elsewhere (4, 5).

EXPERIMENTAL

The following strains of *E. coli* were used: wild type strains K-12 and W; strain M4862, a valine auxotroph derived from strain W; strain M4862-G5, a derivative of strain M4862 which has a second block between both isoleucine and valine and the corresponding α-keto acids (6); and strain 20A19, an isoleucine and valine auxotroph (7). *Aerobacter aerogenes* (strain 1033) was used as a source of acetolactate decarboxylase.

Except for the supplements to the medium that are described in the text the cells were grown in mineral salts-glucose medium. The procedure for the preparation of extracts has also been described previously (6). Each milliliter of the extracts contained the supernatant material from 125 mg. of wet cells after sonic disruption and centrifugation at 28,000 × *g* for 15 minutes. The protein content of such extracts was 15 to 20 mg. per ml. The extract of *A. aerogenes* used as acetolactate decarboxylase was prepared from cells grown aerobically in nutrient broth (Difco) supplemented with 1 per cent glucose.

Acetolactate was determined as acetoin by the method of Westerfeld (8) after decarboxylation by heating in the presence of 1.8 N H_2SO_4. Acetolactate was prepared according to the procedure of Krampitz (9).

RESULTS

Accumulation of Acetoin and Acetolactate by E. coli (strain M4862)—The mutant *E. coli* (strain M4862), a valine auxotroph blocked before α,β-dihydroxyisovalerate, was observed to accumulate acetoin (or diacetyl) under certain cultural conditions. As shown in Table I, the accumulation followed a pattern similar to that noted for most metabolites which precede blocked reactions, *i.e.* when valine was present in excess, the accumulation was suppressed (10). In view of the postulated role of α-acetolactate in valine biosynthesis, it seemed likely that this mutant was blocked between acetolactate and α,β-dihydroxyisovalerate and that acetoin arose from the spontaneous breakdown of acetolactate. Indeed, in culture fluids obtained shortly after valine exhaustion, the acetoin content was considerably increased by acidifying and heating, a treatment which decaboxylates acetolactate.

In order to obtain a sufficient amount of acetolactate to convert to its osazone, strain M4862 was grown in 1 l. of minimal medium supplemented with a limiting amount of L-valine. Incubation with shaking was continued after growth had ceased until the glucose had disappeared. At this time, the culture was centrifuged and phenylhydrazine reagent (4 gm. of phenylhydrazine hydrochloride and 6.4 gm. of sodium acetate dissolved in 30 ml. of water) was added to the supernatant. The osazone that formed was removed by filtration and purified following the procedure used by Juni (11). The product melted at 258–259° (uncorrected) using a Fisher-Johns melting point block, and there was no depression upon mixing with the derivative prepared from synthetic α-acetolactate.

Formation of Acetolactate by Cell-free Extracts—In order to study the mechanism of formation of acetolactate, extracts of the mutant were prepared using cells harvested at a time when the rate of acetolactate formation was high. This condition was achieved by use of the minimal medium supplemented with a limiting amount of L-valine (15 μg. per ml.) and incubating beyond the time of growth factor exhaustion.

In preliminary experiments considerable amounts of acetolactate were formed from pyruvate by such extracts. As shown in Table II, the reaction was markedly stimulated by the addition of TPP[1] and Mg^{++} or Mn^{++}. Adenosine triphosphate was not required for the condensation.

In order to demonstrate that the compound formed by such an extract was indeed acetolactate, 10 ml. of reaction mixture containing 400 μmoles of pyruvate, 100 μg. of TPP, 4.0 ml. of crude extract, 100 μmoles of $MgCl_2$, and 1 mmole of potassium phosphate, pH 8.0, was incubated for 200 minutes at 37°. The reaction was stopped with 1.0 ml. of 10 per cent zinc sulphate and 0.5 ml. of 1 N sodium hydroxide. After centrifugation the supernatant was treated with phenylhydrazine, and the product was purified and identified as the bis-phenylhydrazone of diacetyl as described above.

In agreement with the observations of Juni (11), the extracts

* Supported in part by Grant 4015 from the United States Public Health Service and by funds received by Harvard University from the Eugene Higgins Trust.

[1] The abbreviation used is: TPP, thiamine pyrophosphate.

TABLE I

Growth and acetoin formation by valine auxotroph, Escherichia coli (strain M4862)

Each flask contained 20 ml. of mineral salts-glucose medium supplemented with L-valine as indicated and was incubated with shaking at 37° for 24 hours. Turbidity was determined with a Klett-Summerson colorimeter with a K-42 filter. Acetoin plus diacetyl was determined on supernatant solutions.

L-Valine	Turbidity	Acetoin plus diacetyl
μg./ml.		*μmoles/ml.*
5	81	0.33
12	176	0.97
16	206	1.16
20	252	0.59
30	264	0.19
40	260	0.00

TABLE II

Formation of acetolactate by Escherichia coli extract

Each tube contained in 1.0 ml.: potassium phosphate, pH 8.0, 100 μmoles; sodium pyruvate, 10 μmoles; 0.2 ml. of crude extract from *E. coli* (strain M4862); and where indicated: TPP, 20 mg.; adenosine triphosphate, potassium salt, 2.5 μmoles; $MgSO_4$ or $MnSO_4$, 1 μmole. After incubation for 10 minutes, the reaction was stopped and the acetolactate was decarboxylated with 0.1 ml. of 50 per cent trichloroacetic acid.

Additions	Acetolactate plus acetoin
	μmoles
None	0.19
TPP	0.64
TPP, ATP	0.48
TPP, Mg^{++}	0.92
TPP, Mn^{++}	0.78

of *E. coli* were unable to decarboxylate acetolactate. Therefore it was necessary to convert acetolactate to acetoin chemically in order to employ the color test of Westerfeld (8). The absence of a decarboxylase is compatible with the view that the acetoin formed in cultures of the mutant strain M4862, was actually derived from acetolactate by spontaneous breakdown rather than by a direct action of the organism.

Effect of pH on Acetolactate Formation—In Fig. 1 is shown the effect of pH on acetolactate formation by an extract of strain M4862. Greatest activity was observed at pH 8. Since phosphate buffer was employed in this experiment higher pH values could not be tested. In the presence of tris(hydroxymethyl)aminomethane buffer, activity was much lower, but it is of interest to note that more activity was observed at pH 8.5 than at pH 8.17.

Effect of TPP—As shown in Table II, even without dialysis, the crude extract was highly resolved with respect to TPP. Fig. 2 shows that high concentrations of TPP would be required for maximal activity. Half-maximal activity was obtained only after the addition of 20 μg. of TPP per ml. This high requirement makes it impractical to achieve optimal activity in routine assays.

Nature of Acetolactate Formed—Since Juni and Heyme (12) observed the formation of racemic acetolactate by a pyruvic oxidase preparation from *E. coli*, it was of interest to examine

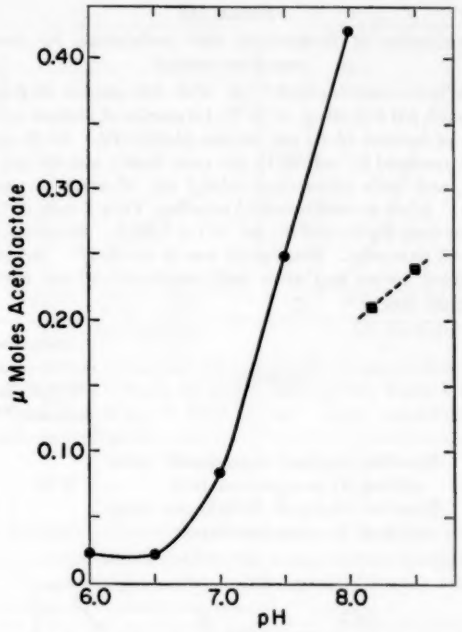

FIG. 1. Effect of pH on acetolactate formation by *Escherichia coli* (strain K-12) extract. Each tube contained 1.0 ml. of reaction mixture; sodium pyruvate, 10 μmoles; $MgCl_2$, 10 μmoles; 40 mg. TPP per ml.; 0.3 ml. of crude extract; ●——● phosphate buffer, 0.10 M; ■——■ tris(hydroxymethyl)aminomethane buffer, 0.10 M. The reaction mixture was incubated in air at 37° for 20 minutes. The reaction was stopped with 0.1 ml. of 10 per cent $ZnSO_4$ and 0.1 ml. of 1 N NaOH. Acetolactate was determined as described in the text. The pH indicated was that of the buffer.

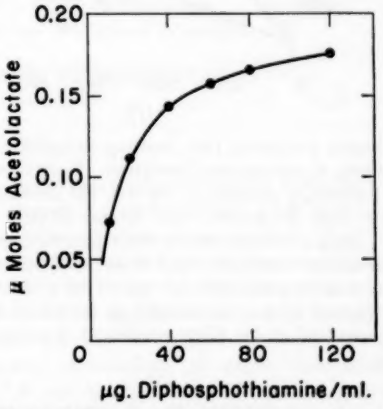

FIG. 2. Effect of TPP on acetolactate formation. Each tube contained in 1.0 ml.: potassium phosphate, pH 8.0, 100 μmoles; sodium pyruvate, 10 μmoles; 0.2 ml. of crude extract from *Escherichia coli* (strain M4862); $MgCl_2$, 10 μmoles; TPP as indicated. Other conditions are as shown or as in Fig. 1.

the nature of the acetolactate formed in the system described here. Crude extracts of *A. aerogenes* (strain 1033), like the strain used by Juni (11), grown so as to contain large amounts of the classical acetoin-forming system (carboligase), will break down only 50 per cent of a synthetic (racemic) preparation of acetolactate. In addition, at pH 8.0, which is optimal for acetolactate

TABLE III

Decarboxylation of Escherichia coli acetolactate by Aerobacter aerogenes extract

Each tube contained: 0.7 ml. with 100 μmoles of potassium phosphate, pH 8.0; 40 μg. of TPP; 4.5 μmoles of sodium pyruvate; 0.1 ml. of extract of *E. coli* (strain M4862-G5). At 30 minutes, Tube 1 received 0.1 ml. of 10 per cent $ZnSO_4$ and 0.1 ml. of 1 N NaOH, and both tubes received 0.3 ml. of crude *A. aerogenes* extract. After an additional 15 minutes, Tube 2 received 0.1 ml. of 10 per cent $ZnSO_4$ and 0.1 ml. of 1 N NaOH. Blank tubes contained no pyruvate. Incubation was in air at 37°. Acetoin was determined before and after acid treatment by the method of Westerfeld (8).

Tube	Conditions	μMoles of acetoin	
		Without acid treatment	With acid treatment
1	Reaction stopped immediately after adding *A. aerogenes* extract.....	0.08	1.49
2	Reaction stopped 15 minutes after adding *A. aerogenes* extract.....	1.16	1.32

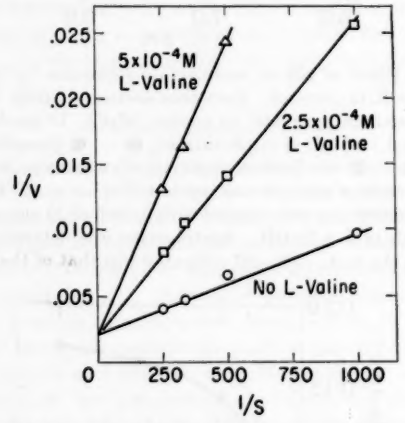

FIG. 3. Double reciprocal plot showing competitive nature of valine inhibition of acetolactate formation. Each tube contained in 1.0 ml.: potassium phosphate, pH 8.0, 100 μmoles; 0.2 ml. of crude extract from *Escherichia coli*, (strain 20A19); $MgCl_2$, 1.0 μmole; TPP, 20 μg.; L-valine and sodium pyruvate as indicated. The reaction mixture was incubated in air at 37° for 10 minutes. The reaction was stopped with 0.1 ml. of 18 N H_2SO_4. Acetolactate determined on 0.5 ml. samples as described in the text. $1/V$ is the reciprocal of the Klett reading; $1/S$ is the molarity^{-1} of pyruvate.

formation by *E. coli* extracts, the *A. aerogenes* extracts will break down but will not form acetolactate. Therefore, an *E. coli* extract was employed to form acetolactate at pH 8.0, and then the product was tested by adding directly the crude *A. aerogenes* extract. As Table III shows, all the acetolactate formed by the *E. coli* extract was broken down, as completely by the enzyme as by acid. It can therefore be concluded that the system studied here forms acetolactate of one optical configuration.

Effect of Valine on Action of Acetolactate-Forming System—The data of Table I show that in the presence of excess valine (*e.g.* the medium containing initially 40 μg. per ml.) acetolactate did not appear in the culture fluids. This effect of end product could be caused by interference with the formation of the acetolactate-forming system, with its action, or with both. Examination of the extracts revealed that L-valine did inhibit the action of the enzyme. Fig. 3 shows that the inhibition by valine is competitive with respect to pyruvate.[2]

TABLE IV

Effect of L-valine on formation of enzyme

Minimal medium, supplemented as indicated in the text, inoculated with a stationary phase culture of *Escherichia coli* (strain M4862-G5) and incubated at 37° with shaking. After a lag period of about 30 minutes, growth began slowly. The culture was divided after 2 hours, one aliquot receiving excess L-valine (100 μg. per ml.). After 1 hour, turbidity was determined and samples were harvested for preparation of extracts.

Extract	Conditions	Turbidity	Total enzyme activity
			μmoles/l./hr.
I	Initial culture	98	105
II	Same, at 2 hrs.	141	762
III	Unmodified aliquot, at 3 hrs.	179	1710
IV	Aliquot with excess valine, at 3 hrs.	200	938

Effect of Valine Deficiency and Excess on Formation of Enzyme System—From the first, the most active extracts were obtained from valine auxotrophs grown on limiting valine. The wild strain grown with excess valine had much less activity. These observations suggested that in addition to suppressing the action of the enzyme as described above, L-valine also suppressed the formation of the enzyme.

The effect of valine on enzyme formation was demonstrated more directly in an experiment in which the double mutant, *E. coli* (strain M4862-G5), was grown from a heavy inoculum under conditions where the entry of valine was rate-limiting.[3] These conditions were achieved by supplementing the minimal medium with 20 μg. of L-valine per ml. and with an excess of L-leucine and L-isoleucine (200 mg. of each per ml.), which would be expected to impede the permeation of valine (13). Thus, valine, though available for protein synthesis, might not reach the intracellular concentration required to suppress formation of the acetolactate-synthesizing enzyme system. As a result the enzyme should be preferentially formed.

The results are shown in Table IV. After 2 hours of incuba-

[2] It may be well to emphasize the dissimilarity in structure of substrate and inhibitor in the example of competitive antagonism described here. Compounds of such dissimilar structure would probably not be selected in any rational search for antagonistic pairs guided by our current concepts of the stereochemical basis of competitive inhibition. Conceivably, one compound could serve as an effective, competitive inhibitor of a second if it masked only a part of the reactive site on the enzyme. Although there is ample justification for the continued systematic testing of structural analogues for possible chemotherapeutic value, the examples of feedback control that have been described indicate that a systematic testing of randomly selected compounds might be equally rewarding.

[3] The additional requirement for isoleucine in this double mutant did not influence the results. The same kind of results would have been obtained if the parent mutant had been used.

tion only a little growth had occurred but the acetolactate-synthesizing system had increased 7-fold. At this time the culture was divided and to one aliquot an excess (100 μg. per ml.) of L-valine was added. During an additional hour of incubation the cells in the untreated aliquot increased by 27 per cent, whereas the enzyme activity increased by 124 per cent. In contrast, the aliquot receiving excess valine showed a greater amount of cell synthesis but essentially no further enzyme formation.[4]

Effect of Enzyme Concentration—In Fig. 4, Graph A shows the effect of extract volume exhibiting relatively low activity (Extract I in Table IV). A virtually linear increase in acetolactate formation with increase in concentration of extract was observed. Extracts exhibiting high acetolactate-synthesizing activity, obtained from mutants when valine was the factor limiting growth, were usually employed in this study. These extracts, however, showed anomalous behavior when the effect of extract volume was examined. Thus, in Fig. 4, Graph B shows that one such active extract (Extract III, Table IV) exhibited activity which increased exponentially with extract concentration. In order to make an estimate of the activity of such extracts a line was drawn in the region of the curve that appears nearly linear. For example, in Graph B the activity of the extract was determined from the slope of the constructed Line a-----b.

This peculiar property of active extracts was not altered by increasing TPP or Mg^{++} levels or by the addition of a boiled extract. Changing the pH or the length of incubation also failed to eliminate the effect. Quite possibly, at low extract concentrations the first of a pair of enzymatic steps (perhaps the generation of an active acetal group) is limiting. However, no evidence for more than one step has been found in preliminary attempts to fractionate the system.

Acetolactate Formation in Wild type E. coli—In order to eliminate the possibility that the formation of acetolactate was some sort of adaptively formed mechanism peculiar to valine auxotrophs, *E. coli* (wild type strains W and K-12) was examined for the capacity to form acetolactate when grown in minimal medium. Table V shows that both strains exhibit an acetolactate-synthesizing activity which is inhibited by L-valine. The table also shows that when the cells were grown in the presence of excess L-valine the formation of the enzyme was suppressed. These results indicate that the same system is found in the wild type and in the mutants that were blocked at late steps in valine biosynthesis.

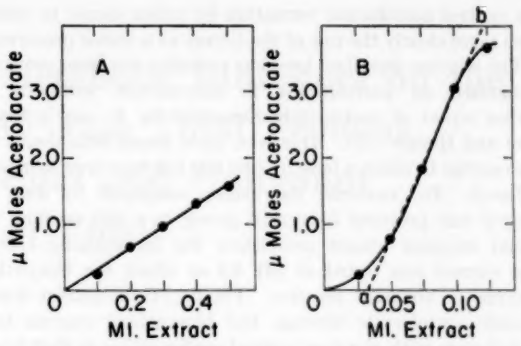

FIG. 4. Effect of extract concentration on acetolactate formation: *A*, Extract I, Table III and *B*, Extract III, Table III. Each tube contained 40 μg. of TPP per ml. Other conditions are as shown or as in Fig. 1.

TABLE V

Acetolactate formation by wild types of Escherichia coli

Each extract was tested in the assay system as described in Fig. 1, except for the addition of 80 μg. of TPP and, where present, 5 μmoles of L-valine per tube.

Extract prepared from	Specific activity (μmoles of acetolactate/gm. of wet cells/hr.)	
	Test without valine	Test with valine
E. coli (strain K-12) grown in minimal medium	129	24
E. coli (strain K-12) grown in minimal medium + valine + isoleucine	45.7	
E. coli (strain W) grown in minimal medium	60	7.7
E. coli (strain W) grown in minimal medium + valine	9.2	2.0

DISCUSSION

It has been reported previously from this laboratory (4) that acetolactate-3-C^{14} is incorporated into valine of *E. coli* proteins. Furthermore, acetolactate, like other 5-carbon precursors of valine, produces an inhibition of growth of *E. coli* (strain K-12). More recently, the conversion of acetolactate to an α-ketoisovalerate by yeast enzymes in the presence of triphosphopyridine nucleotide has been reported by Strassman *et al.* (14, 15). Experiments recently reported from this laboratory (16) indicated that acetolactate and the corresponding isoleucine precursor, α-aceto-α-hydroxybutyrate (17), underwent reduction in the presence of an enzyme, Mg^{++}, and reduced triphosphopyridine nucleotide to form products tentatively identified as the dihydroxy precursors of valine and isoleucine.

The observations reported here greatly strengthen the view that acetolactate can not only be converted to valine but is an obligatory intermediate in valine biosynthesis. Thus a mutant of *E. coli* blocked before dihydroxyisovalerate accumulated acetolactate. An enzyme that forms acetolactate from pyruvate was found in extracts not only of this mutant but also of wild type organisms.

As observed with other examples of biosynthetic enzymes, when the wild type was grown under conditions in which valine synthesis was not required for growth, *i.e.* when L-valine had been added to the minimal medium, the formation of the enzyme was suppressed. Furthermore, in common with the initial steps leading to isoleucine (6), to proline (18), and to pyrimidines (19), the action of this enzyme was inhibited by the end product, L-valine. The physiological significance of negative feedback loops controlling biosynthesis is now well recognized. Therefore,

[4] The extract prepared from cells harvested from the medium containing excess L-valine might conceivably owe its lower activity to the presence of free valine in the extracts. This possibility cannot account for much of the depression since dialysis of such extracts did not increase the activity by more than 10 per cent.

the control acetolactate formation by valine seems to indicate even more clearly the role of the former as a valine precursor.

The enzyme described here has probably not been previously recognized as participating in acetolactate synthesis. An earlier report of acetolactate formation by *E. coli* is that of Juni and Heyme (12). However, there seems little doubt that the enzyme to which a biosynthetic role has here been ascribed is different. For example, the extract employed by Juni and Heyme was prepared from cells grown in a rich medium that would suppress almost completely the biosynthetic enzyme. The extract was tested at pH 5.9 at which the biosynthetic enzyme is virtually inactive. Finally, their extract formed racemic acetolactate whereas the biosynthetic enzyme forms acetolactate with the same optical configuration as that formed and decarboxylated by the classical carboligase of *A. aerogenes* (11).[5]

The question might be raised whether the enzyme described here might not also catalyze the formation of acetohydroxybutyrate. An analogy for this possibility is found elsewhere in the synthesis of valine and isoleucine, since a single enzyme catalyzes the formation of both keto acids from the corresponding dihydroxy acids, and the same is true of the amination of both keto acids. This question has not yet been answered since the conditions required for the formation of acetohydroxybutyrate from pyruvate and α-ketobutyrate would also yield acetolactate, and the analytical method used here would not distinguish the two compounds.[6] This problem and the nature of the remaining steps in isoleucine and valine biosynthesis are currently under study in this laboratory.

SUMMARY

1. A valine auxotroph, *Escherichia coli* (strain M4862), accumulates α-acetolactate when grown on limiting amounts of valine. The acetolactate spontaneously decomposes in the medium to yield acetoin.

2. An enzyme in wild type *Escherichia coli* forms acetolactate from pyruvate. The enzyme shows a pH optimum in phosphate buffer of 8.0 or higher, is markedly stimulated by Mg^{++}, and requires a high concentration of thiamine pyrophosphate.

3. The formation of the enzyme is depressed by excess valine and increased under conditions where valine is growth-limiting. Valine also inhibits the action of the enzyme, and this inhibition is competitive with respect to pyruvate.

4. These observations provide strong evidence that acetolactate is an intermediate in valine biosynthesis. The effects of valine on the formation and on the action of the enzyme thus serve to regulate the biosynthesis of valine.

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[5] In addition to the classical carboligase with its catabolic function, *A. aerogenes* has also been shown (5) to have the biosynthetic acetolactic-forming system. A comparison of these two enzymes catalyzing the same reaction for two different biological functions in this organism will be described in a future communication.

[6] If only one enzyme were present, valine would block not only its own synthesis but isoleucine synthesis as well, and hence would prevent growth unless isoleucine or one of its 6-carbon precursors were supplied. Since valine is not normally an inhibitor of growth, it may be inferred that separate enzymes catalyze the formation of acetolactate and acetohydroxybutyrate. An exception is found in the K-12 strain of *E. coli* which is inhibited by valine. Possibly, one enzyme catalyzes both steps in this strain.

Effects of Various Steroids and Metabolic Inhibitors on the Incorporation of Glycine-2-C^{14} into Total Proteins and Nucleic Acids of Normal and Malignant Lymphocytes *in Vitro**

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(Received for publication, June 24, 1958)

The established roles of lymphoid tissue in a variety of biological phenomena (1), including antibody formation and heterologous tissue transplantation, have focused attention on the metabolism of lymphoid cells. Demonstration that the lymphocyte is an end cell of action of certain adrenal cortical steroids (2, 3), and production (4) of cytological alterations in lymphocytes *in vitro* by addition of exceedingly low concentrations of cortisone (10^{-7} M) have suggested studies of the effects of steroids on lymphocyte metabolism.

A number of different steroids, added to suspensions of lymphocytes prepared from rat thymus or from a transplantable lymphosarcoma, inhibit glucose oxidation, oxygen consumption, and endogenous respiration characteristic of these cells (5). The low concentrations (10^{-5} M) at which the steroids were effective and the fact that similar alterations in metabolism were observed in lymphocytes from rats given steroid by injection 4 hours before being killed (5) indicated some physiological significance for the phenomena studied.

These observations, together with the indicated roles of lymphoid tissue in protein metabolism (6) and its high content of nucleoprotein, have led to a study of protein and nucleic acid formation in isolated normal and malignant lymphocytes and a consideration of the influence of certain steroids and metabolic inhibitors on these processes. Allfrey *et al.* (7) have examined in some detail the incorporation of labeled amino acids into the isolated nuclei of calf thymic lymphocytes. Kit and Barron (8) compared protein synthesis *in vitro* by a variety of lymphoid cells and studied the effects of hydrocortisone and cortisone on certain of the metabolic processes in these cells.

EXPERIMENTAL

Animals—Male rats (Sprague-Dawley strain)[1], of weights ranging between 140 and 200 gm., were the source of lymphoid tissue. Animals were killed by decapitation and thymus or transplantable Murphy-Sturm lymphosarcoma[2] was rapidly removed by dissection. Lymphosarcoma tissue was removed 12 to 14 days after subcutaneous transplantation into the host; at this time tumors were still in a phase of rapid growth. Adrenalectomized rats were used 2 to 3 weeks after bilateral adrenalectomy. All animals received Rockland rat pellets and water *ad libitum* except for the adrenalectomized rats which were given 1 per cent saline as drinking fluid. In steroid experiments *in vivo*, intraperitoneal injections of steroids[3] as fine suspensions in isotonic saline (5 mg. of steroid per 100 gm. of body weight) were given at 24 and again at 4 hours before decapitation of the animals. To label lymphoid tissue *in vivo*, 0.1 mc. of DL-lysine-2-C^{14} (30.1 mg. of the hydrochloride)[4] or 0.1 mc. of DL-phenylalanine-3-C^{14} (19.8 mg. of the hydrochloride)[4] in 1.0 ml. of isotonic saline was given by intraperitoneal injection 10 hours before the animal was killed.

Cell Suspensions—Suspensions of washed thymic lymphocytes or of tumor cells were prepared essentially in the manner described by Farber *et al.* (9) in a modified Krebs-Ringer phosphate or phosphate-bicarbonate buffer, pH 7.4, but with the procedure modified as previously described (10). Tumor cell suspensions were prepared by a modification of a procedure designed by Kaltenbach (11) for liver cell suspensions. Minced tumor tissue was gently forced through a 14 mesh, Monel metal screen[5] with a clean, soft rubber stopper while the tissue was being washed with cold buffer. The process was repeated with the use of a 24 mesh, Monel metal screen but with no application of pressure or agitation. All operations were conducted at 0°. Cells were washed with buffer by centrifugation[6] at 1000 r.p.m.

* This study was aided by grants (Nos. P-68, 350 (A) and (B), and CY-3154, respectively) from the American Cancer Society, the Damon Runyon Memorial Fund for Cancer Research, Inc., and the National Institutes of Health, United States Public Health Service.

[1] Purchased from the Holtzman Rat Company, Madison, Wisconsin.

[2] Grateful acknowledgment is made to Dr. K. Sugiura, the Sloan-Kettering Institute for Cancer Research, New York, for providing the initial lymphosarcoma used for transplantation in these studies.

[3] The steroids used were generously provided as follows: cortisone, hydrocortisone, and corticosterone, by Merck, Sharp and Dohme; deoxycorticosterone, by the Schering Corporation; 17α-methyltestosterone, 17α-ethyltestosterone, and 19-nortestosterone, by Chemical Specialties Company, Inc.; 17α-ethyl,19-nortestosterone, by G. D. Searle and Company; and testosterone, by Sterol Derivatives, Inc.

[4] Obtained from Tracerlab, Inc., Boston, through allocation by the Atomic Energy Commission.

[5] Whitehead Metal Products Company, New York.

[6] All centrifugations described in this paper were conducted at 0° in an International refrigerated centrifuge, model PR-2.

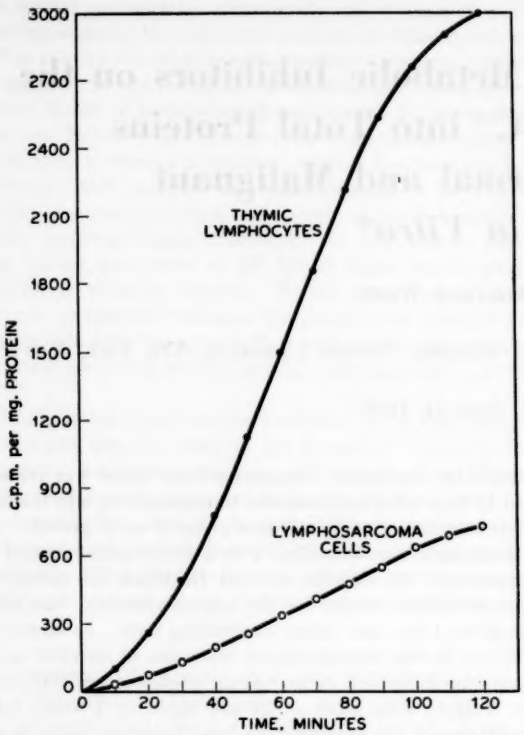

FIG. 1. Time course of glycine-2-C^{14} incorporation into the proteins of suspensions of rat thymic lymphocytes and lymphosarcoma cells. In this and in subsequent figures and tables, protein specific activities have been corrected to a unit number (10^9) of cells.

and were finally suspended in an equal volume of buffer for use in incubations.

Cell counts were made in a hemocytometer on diluted aliquots of suspensions, with 1:4000 Safranin O in Tyrode's solution (12). Thymic and tumor cell suspensions contained approximately 1.5×10^9 and 0.5×10^9 cells per ml., respectively. Dry weight determinations showed that 10^6 thymic or lymphosarcoma cells weighed approximately 32 and 46 μg., respectively.

Incubation Procedure—Incubations were conducted in Warburg vessels which contained, unless otherwise indicated, 2 to 8×10^6 cells, 20 μmoles of glucose, 8 μmoles of glycine-2-C^{14} (1.0 μc.),[7] and modified Krebs-Ringer phosphate or phosphate-bicarbonate buffer, pH 7.4, to make a final volume of 2.0 ml. Metabolic inhibitors were added in aqueous solutions buffered between pH 7.0 and 7.4. Steroids were added in alcoholic solution to appropriate vessels, with subsequent evaporation of solvent by a gentle stream of warm air. Steroid concentrations were well below their maximal solubilities in buffered salt solutions (13, 14). In aerobic studies the gas phase was air, and anaerobic conditions consisted of 95 per cent nitrogen-5 per cent carbon dioxide. Unless otherwise noted incubations continued for 2 hours at 37°. In oxygen uptake studies, filter paper moistened with 0.2 ml. of 20 per cent potassium hydroxide was present in the center well of the flasks to absorb carbon dioxide. Respiratory activities are reported as Q_{O_2}, microliters of oxygen taken up per hour per mg. dry weight of tissue. Anaerobic glycolytic activities are reported as the carbon dioxide equivalent of lactic acid production or Q_{CO_2}, microliters of carbon dioxide released per hour per mg. dry weight of tissue.

Isolation and Radioactivity Counting Procedures—Incubations were terminated by addition of an equal volume of cold 20 per cent trichloroacetic acid which contained 0.8 M nonradioactive glycine. The trichloroacetic acid-insoluble material was washed and lipide was extracted according to Winnick *et al.* (15). A nucleic acid fraction was next obtained by the procedure of Schneider (16); aliquots of such fractions, after removal of trichloroacetic acid, were plated directly in stainless steel planchets for counting. Samples of purified proteins were suspended by homogenization (9) and deposited on filter paper circles (7) for counting in a windowless gas flow counter; counts were corrected for self-absorption. Activities of protein samples are expressed as counts per minute per mg. of protein and nucleic acid samples as total counts per minute, each being corrected to a unit number of cells, *viz.* 10^9.

RESULTS

Time Course of Glycine-2-C^{14} Incorporation—When suspensions of rat thymic lymphocytes were incubated in air in the presence of glucose and glycine-2-C^{14}, there was a rapid incorporation of isotope into cellular proteins and nucleic acids. Fig. 1 shows the time course of incorporation of glycine-2-C^{14} into total proteins. After an initial "lag" period of 10 to 20 minutes, the C^{14} uptake proceeded linearly for approximately 90 minutes and then began to decline in rate. Incorporation of C^{14} into proteins of lymphosarcoma cells, under these conditions, proceeded at a rate which was only about 25 per cent of that observed with normal thymic cells (Table I). Plotted data representing glycine-2-C^{14} incorporation into mixed nucleic acids of thymic lymphocytes are similar to those shown in Fig. 1 for proteins.

Evidence for Synthesis of New Molecules versus Exchange Phenomena—Incorporation of glycine-2-C^{14} into the protein of thymic cells was allowed to proceed until the time indicated by the arrow in Fig. 2. Lymphocytes were then separated by centrifugation, and isotopic glycine was removed by washing with buffer until the washings contained no radioactivity. Ali-

TABLE I

Relative rates of incorporation in vitro of glycine-2-C^{14} into total protein and nucleic acids of lymphosarcoma and thymic cell suspensions

Cells	Aerobic			Anaerobic		
	Proteins	Nucleic acids	Q_{O_2}	Proteins	Nucleic acids	Q_{CO_2}
Thymus, intact rats*	100	100	7.3	7	7	9.0
Thymus, adrenalectomized rats	78	80	7.0			
Lymphosarcoma, intact rats	23	37	2.2	117	117	32
Thymus, lymphosarcoma-bearing rats	77	75	6.5			

* Rates of incorporation for normal thymic lymphocytes under aerobiosis are used as the reference standards and are arbitrarily given as 100 in this table.

[7] Obtained from the New England Nuclear Corporation, Boston, through allocation by the Atomic Energy Commission.

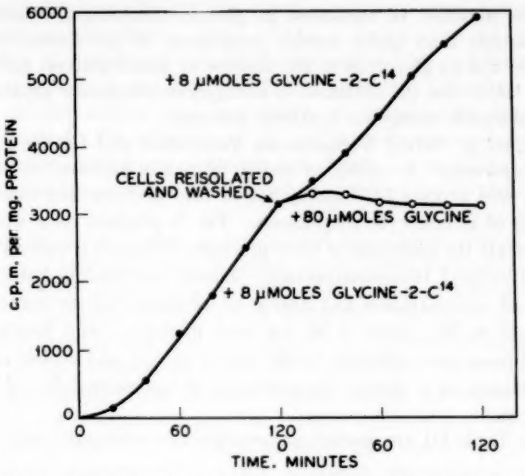

FIG. 2. Demonstration of the nonexchangeability of incorporated glycine-2-C^{14}.

quots of these cells were reincubated with a further increment of isotopic glycine or with a 10-fold excess of nonlabeled glycine. It is evident from the upper curve in Fig. 2 that reisolation of the cells did not affect their ability to continue incorporation of labeled glycine. It can also be seen that the specific activity of cellular protein remained essentially constant for 2 hours even in the presence of a large excess of unlabeled glycine; the slight rise in the curve may represent incorporation of intracellular glycine-2-C^{14} not removed by the washing procedure (*cf.* Fig. 5). The same relationships obtained in the case of the nucleic acids. Furthermore, after thymic cells were labeled *in vivo* by intraperitoneal administration of DL-phenylalanine-3-C^{14} and DL-lysine-2-C^{14}, such cells were studied *in vitro* as described above; results qualitatively similar to those described in the glycine studies were obtained. Specific activities of representative samples of purified, radioactive proteins were unchanged after treatment with performic acid, mercaptoethanol, hot water, and dilute alkali according to the procedures of Peterson and Greenberg (17).

Protein samples (10 mg.) were partially hydrolyzed by treatment with 10 ml. of 6 N hydrochloric acid at 37° for 7 days. Five radioactive peptides were isolated by ascending chromatography on Whatman No. 1 filter paper in a system of *n*-butanol-acetic acid-water (18), and each was then completely hydrolyzed (6 N hydrochloric acid at 105° for 18 hours); hydrolysates were subjected to two-dimensional chromatography by means of the system described above and a 2,6-lutidine-2,4,6-collidine-water-diethylamine system (19). Radioactivity of isolated peptides was the result only of their content of radioactive glycine and serine.

Relative Rates of Incorporation—Rates of incorporation of glycine-2-C^{14} into cellular proteins and nucleic acids were established over 2-hour periods of incubation; relative rates are summarized in Table I. Q_{O_2} values and Q_{CO_2} values are included; the latter dropped to zero when glucose was omitted from the medium. Adrenalectomy or the presence of a growing tumor in the host, decreased by 20 to 25 per cent the rate of glycine incorporation aerobically by thymic lymphocytes, and in the case of lymphosarcoma cells synthetic rates were only 25 to 30 per cent of those seen with normal thymic lymphocytes; comparable differences were observed for respiratory rates. Under conditions of anaerobic glycolysis, synthetic rates for thymic lymphocytes were very low, whereas those for lymphosarcoma cells were considerably higher than the reference standards; these differences were also evident in the rates of anaerobic glycolysis.

TABLE II

*Effect of glucose upon glycine-2-C^{14} incorporation into total proteins and nucleic acids of lymphocytes**

Conditions	Thymic cells		Lymphosarcoma cells	
	Proteins	Nucleic acids	Proteins	Nucleic acids
Aerobic				
Without added glucose.....	720	423	63	250
With added glucose†.....	2500	1330	830	780
Anaerobic				
Without added glucose.....	10	58	23	247
With added glucose†.....	203	286	3235	2090

* Values given for protein are counts per minute per mg.; for nucleic acids, total counts per minute. All values are corrected to 10^9 cells.

† Final glucose concentration was 0.01 M.

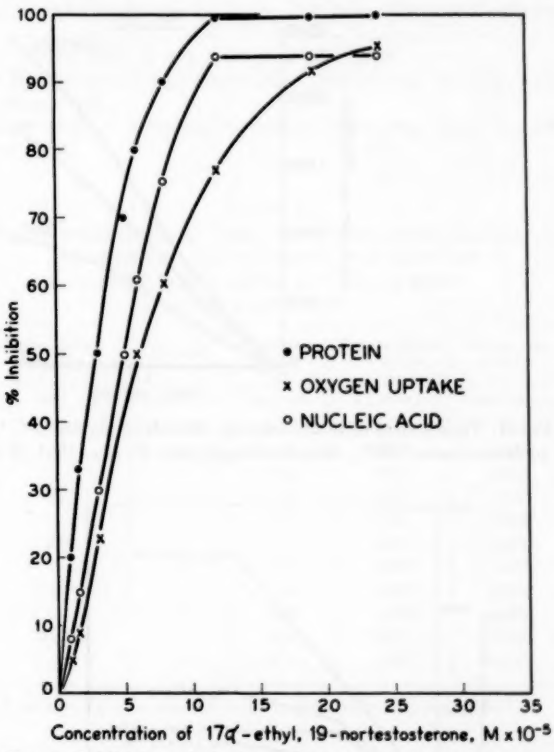

FIG. 3. Inhibition of glycine-2-C^{14} incorporation into proteins and nucleic acids of thymic lymphocytes and inhibition of concomitant respiration as a function of the concentration of 17α-ethyl,19-nortestosterone.

Role of Glucose in Glycine-2-C^{14} Incorporation—Incorporation reactions exhibited a glucose dependency which was most striking when energy for anabolic processes was provided chiefly

TABLE III

Effect of steroids upon respiration and glycine-2-C¹⁴ incorporation into proteins and nucleic acids of thymic lymphocytes

Steroid	Concentration of steroid (M × 10⁻⁶) required to produce 50% inhibition		
	Oxygen uptake	Incorporation into protein	Incorporation into nucleic acids
17α-Ethyl,19-nortestosterone	6.2	3.2	5.2
17α-Ethyltestosterone	9.0	5.3	10
Deoxycorticosterone	17	5.0	8.5
17α-Methyltestosterone	17	15	11
19-Nortestosterone	42	14	27
Corticosterone	36	16	25
Testosterone	70	36	31
Hydrocortisone	143	57	70
Cortisone	192	120	96

more sensitive to *variations* in glucose concentrations under anaerobic than under aerobic conditions; in the former case there was no glycolysis in the absence of added glucose, and in the latter case the oxidation of endogenous substrates[8] provided considerable energy for synthetic processes.

Effect of Steroid Hormones on Respiration and Glycine-2-C¹⁴ Incorporation—A variety of steroid hormones inhibited respiration and glycine-2-C¹⁴ incorporation into proteins and nucleic acids of lymphocyte suspensions. Fig. 3 presents data which illustrate the inhibition of these processes in thymic lymphocytes by 17α-ethyl,19-nortestosterone. Linear relationships between steroid concentration and degree of inhibition obtain approximately to the point of 80 per cent inhibition, and maximal inhibitions were attained, in the case of protein and nucleic acid syntheses, at a steroid concentration of approximately 1.2×10^{-4} M.

In Table III are assembled concentration-inhibition data for

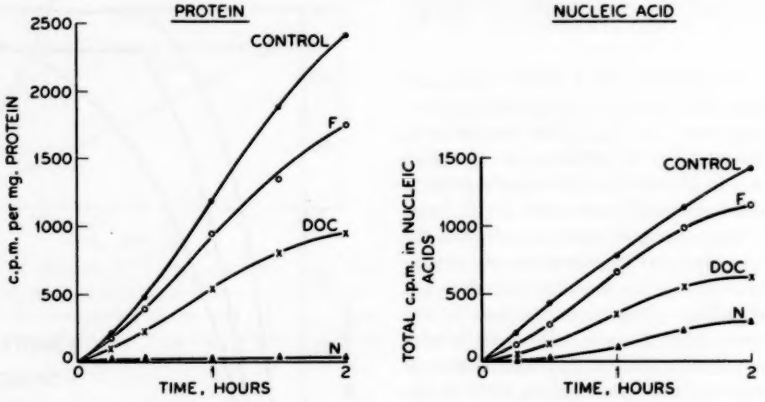

FIG. 4. Time course of inhibitions by steroids of glycine-2-C¹⁴ incorporation into proteins and nucleic acids of thymic lymphocytes. *F*, hydrocortisone; *DOC*, deoxycorticosterone; *N*, 17α-ethyl,19-nortestosterone.

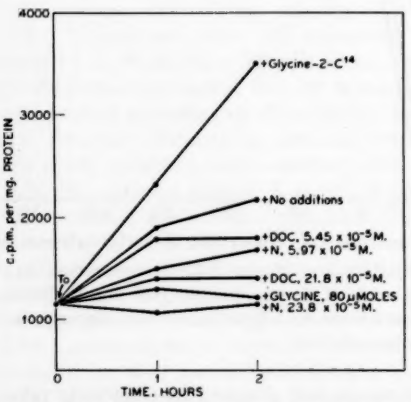

FIG. 5. Effect of added steroids upon the further incorporation of intracellular radioactive glycine by thymic lymphocytes previously labeled *in vitro* with glycine-2-C¹⁴. *To*, initial protein specific activity of labeled lymphocytes; *DOC*, deoxycorticosterone; *N*,17α-ethyl,19-nortestosterone.

by anaerobic glycolysis of glucose (Table II). Previous observations[8] had shown that the incorporation reactions were much

[8] M. Blecher, and A. White, unpublished observations.

a variety of steroids. The steroids tested varied greatly in their inhibitory capacities. There was an evident correlation, in most instances, between the effectiveness of a steroid in diminishing oxygen uptake and inhibition of protein and nucleic acid syntheses. Steroid effects were qualitatively and quantitatively similar in studies with lymphosarcoma cell suspensions.

Similar results were obtained when 17α-ethyl,19-nortestosterone, deoxycorticosterone, or hydrocortisone were administered to normal or adrenalectomized rats (see "Experimental"). Thymic lymphocytes from such animals exhibited degrees of inhibition of respiration and of glycine incorporation similar to those observed when the steroids were added to cell suspensions *in vitro*.[9] Of the steroids given by injection, only hydrocortisone induced thymic involution, as determined by weight loss and gross appearance.

Time Course of Steroid Inhibitions—Thymic lymphocytes were incubated aerobically with glycine-2-C¹⁴ in the presence of glucose; each steroid, when added, was present in a concentration of 10^{-4} M. Inhibition of protein or nucleic acid synthesis became apparent within the first few minutes of incubation (Fig.

[9] While this paper was in preparation, similar observations were reported by Tappan *et al.* (20) who used cortisone and hydrocortisone.

4), and differences in inhibitory potency among steroids were evident at this time.

Effect of Steroids on Labeled Lymphocytes—This experiment was designed to ascertain whether a catabolic influence on protein and nucleic acid metabolism of lymphocytes might contribute to the inhibition of glycine incorporation noted in experiments previously described (*cf.* Table III and Fig. 4).

Thymic lymphocytes were labeled *in vitro* by prior incubation with glycine-2-C^{14} or, *in vivo*, by prior administration of DL-phenylalanine-3-C^{14} and DL-lysine-2-C^{14} (see "Experimental"). Cells were isolated by centrifugation, washed free from extracellular isotopic amino acids and then were incubated with additions as described in Fig. 5 and Table IV. Radioactivity of cellular proteins and nucleic acids, as well as rates of oxygen uptake, were determined at intervals thereafter. As the data in Fig. 5 indicate, the reisolation procedure did not affect the ability of the cells to incorporate further increments of labeled glycine (*cf.* Fig. 2). It is also apparent that considerable intracellular isotopic glycine remained despite the washing procedure; this was reflected in the increase of protein specific activity with time in the absence of additions other than the glucose of the medium (*cf.* Fig. 2). This increase was prevented by reincubation in the presence of steroids. This blocking action by steroids was proportional to steroid concentration and again 17α-ethyl,19-nortestosterone was the most effective (*cf.* Table III). Reincubation in the presence of a large supply of unlabeled amino acid yielded results similar to those presented in Fig. 2. Qualitatively similar results were observed when determinations were made of nucleic acid radioactivity, although less radioactivity appeared in the nucleic acids than in the proteins when glucose was the only supplement to the medium.

When thymic lymphocytes were labeled by prior administration of DL-lysine-2-C^{14} and DL-phenylalanine-2-C^{14}, the amount of such unincorporated amino acids which remained in the cells after the isolation and washing was so small that there was *no* increase in protein or nucleic acid radioactivity during subsequent incubations in the absence of additional labeled amino acid (Table IV). Radioactivity was also unaffected, in this instance, by the presence of 17α-ethyl,19-nortestosterone or high concentrations of nonlabeled lysine and phenylalanine during reincubation.

Effect of Preincubation of Thymic Lymphocytes with Steroids on Subsequent Incorporation of Glycine-2-C^{14}—Thymic cells were incubated with 17α-ethyl,19-nortestosterone or deoxycorticosterone for periods up to 2 hours. Cells were isolated by centrifugation, and steroids were removed by repeated washing with buffer.[10] Cells were then reincubated with glycine-2-C^{14} plus glucose in the usual manner. The data of Table V indicate that 17α-ethyl,19-nortestosterone had not produced irreversible alterations in the respiratory and incorporating systems of the cells. Although the extent of glycine incorporation into proteins and nucleic acids diminished with an increase in the duration of preincubation with steroids, incorporation declined in a manner generally parallel to that observed without steroid (*cf.* Fig. 1). Results similar to those presented in Table IV were also obtained with deoxycorticosterone.

[10] Removal of steroid by this procedure was established in separate experiments. The washed cells were extracted with acetone. Acetone extracts were evaporated to dryness under nitrogen, the residue was taken up in ethanol, and optical density at 240 mμ was measured in comparison with appropriate tissue blanks. No steroid was detected.

TABLE IV

Effect of added steroid upon further incorporation of intracellular radioactive lysine and phenylalanine by thymic lymphocytes previously labeled in vivo with lysine-2-C^{14} and phenylalanine-3-C^{14}

Additions*	Oxygen uptake		Radioactivity: Proteins†		Radioactivity: Nucleic acids‡	
	1 hr.	2 hrs.	1 hr.	2 hrs.	1 hr.	2 hrs.
	μmoles		*c.p.m./mg.*		*total c.p.m.*	
None	7.38	14.8	1340	1425	293	319
17α-Ethyl,19-nortestosterone (2.97×10^{-5} M)	6.17	11.5	1390	1440	285	380
17α-Ethyl,19-nortestosterone (11.9×10^{-5} M)	0.88	1.05	1375	1495	311	357
DL-Lysine (0.08 M) and DL-phenylalanine (0.004 M)	6.76	12.9	1425	1380	305	333
Glycine-2-C^{14} (0.004 M)	7.32	14.0	3000	4500	867	1910

* Glucose (0.01 M) was present in all vessels.

† Specific activity of lymphocyte protein was, initially, 1420 c.p.m. per mg.

‡ Activity of lymphocyte nucleic acids was, initially, 340 total c.p.m.

TABLE V

Effect of preincubaton of thymic lymphocytes with 17α-ethyl, 19-nortestosterone upon subsequent respiration and incorporation of glycine-2-C^{14} into proteins and nucleic acids

Preincubation period	Steroid	Oxygen uptake	Protein specific activity	Nucleic acid activity
min.	1×10^{-4} M	*μmoles/hr.*	*c.p.m./mg.*	*total c.p.m.*
0	−*	8.1	2430	1690
15	−	6.3	2380	1170
	+	6.4	2365	1210
30	−	6.6	1650	1030
	+	7.0	2050	1225
60	−	6.7	1915	1155
	+	6.9	1500	1100
90	−	7.0	1625	1115
	+	6.8	1705	1100
120	−	6.6	1435	1060
	+	6.8	1500	1135

* Minus sign, no steroid added; plus sign, steroid added.

Effect of Metabolic Inhibitors—Substances which are known to inhibit oxygen uptake or to uncouple oxidative phosphorylation have been shown to inhibit incorporation of radioactive amino acids into protein of suspensions of Gardner lymphosarcoma cells (9), of liver homogenates (17), and of isolated calf thymic nuclei (7). The data of Table VI indicate that known uncouplers of oxidative phosphorylation, *viz.* methylene blue (21) and 2,4-dinitrophenol (22), markedly inhibited incorporation of glycine-2-C^{14} into proteins and nucleic acids of thymic lymphocytes at concentrations which did not inhibit oxygen uptake. This is in contrast to the effect of Dicumarol, also reported (23) to uncouple oxidative phosphorylation; Dicumarol markedly inhibited respiration as well as protein and nucleic acid syntheses. Antimycin A (24), Janus green B,

TABLE VI

Effect of metabolic inhibitors upon respiration and incorporation of glycine-2-C^{14} into proteins and nucleic acids of thymic lymphocytes

Inhibitor	Concentration ($M \times 10^{-5}$)	% Inhibition		
		Oxygen uptake	Incorporation into	
			Proteins	Nucleic acids
Antimycin A, 1 μg. per ml		96	95	83
Dicumarol	15	77	99	93
2,4-Dinitrophenol	8	+23*	70	85
	12	10	73	88
	18	30	75	89
	24	47	76	91
Janus green	20	69	99	94
Methylene blue	20	0	35	45
Sodium azide	100	54	88	81
Sodium cyanide	100	9	37	17

* A plus sign indicates an augmentation rather than inhibition.

TABLE VII

Relative effects of 17α-ethyl,19-nortestosterone upon respiration and glycine-2-C^{14} incorporation by thymic lymphocytes in presence and absence of glucose*

Glucose concentration	% Inhibition		
	Q_{O_2}	Incorporation into protein	Incorporation into nucleic acids
None	13	17	26
0.002 M	17	25	22
0.010 M	22	48	40

* 3×10^{-5} M

TABLE VIII

Effect of steroids upon glycine-2-C^{14} incorporation by lymphocytes under conditions of anaerobic glycolysis

Steroid	Concentration	$\left(Q_{CO_2}^{N_2}\right)$		Protein specific activity		Nucleic acid activity	
		TL*	LC†	TL	LC	TL	LC
	$M \times 10^{-5}$	μmoles/hr./mg.‡		c.p.m./mg.		total c.p.m.	
None		9.0	32	133	3800	286	1760
Hydrocortisone	32	5.0	32	28	1330	280	1515
	64	3.0	32	17	1026	210	1020
Deoxycorticosterone	5.0	4.8	32	37	2010	233	1177
	10	3.0	29	19	1785	145	757
17α-Ethyl,19-nortestosterone	3.0	2.4	29	17	1635	94	1072
	6.0	1.4	27	15	1025	68	440

* Thymic lymphocytes.
† Lymphosarcoma cells.
‡ Tissue, dry weight.

and sodium azide, which inhibited oxygen uptake, also had pronounced inhibitory effects upon glycine incorporation. Sodium cyanide, in the concentration tested, had little effect upon the three parameters of metabolism studied. Similar results were obtained in comparable studies with suspensions of lymphosarcoma cells.

Steroid Effects in Absence of Glucose—To determine whether, under aerobiosis, steroids might influence utilization of exogenous glucose, the effects of 17α-ethyl,19-nortestosterone on thymic lymphocytes were studied in the absence and presence of exogenous glucose (Table VII). Inhibitions by this steroid of oxygen uptake and glycine-2-C^{14} incorporation into cellular proteins and nucleic acids were evident in the absence of added glucose, although such inhibitions were somewhat less than those observed in the presence of exogenous glucose. Comparable experiments were not performed under anaerobic conditions since, under such conditions and in the absence of added glucose, protein and nucleic syntheses by thymic lymphocytes are relatively insignificant (Table II).

Steroid Effects during Anaerobic Glycolysis—The possibility existed that the site of action of the steroids might be on the respiratory chain of enzymes and that the former might act by uncoupling oxidative phosphorylation. To determine whether energy production by another metabolic system, *e.g.* anaerobic glycolysis, might also be inhibited by steroids, the effects of a number of steroid hormones on the rates of anaerobic glycolysis and concomitant incorporation of glycine-2-C^{14} into proteins and nucleic acids were studied in thymic lymphocytes and in lymphosarcoma cells (Table VIII). In the case of thymic lymphocytes, anaerobic glycolysis as well as glycine incorporation were inhibited by each of the three steroids tested. With lymphosarcoma cells, glycine incorporation was markedly inhibited by each of the three steroids, whereas rates of anaerobic glycolysis remained relatively unaffected. As was the case in aerobiosis (Table III), 17α-ethyl,19-nortestosterone was the most potent steroid under anaerobic conditions.

DISCUSSION

An experimental procedure has been described which permits study of factors which influence the incorporation of the radioactivity of glycine-2-C^{14} into the total proteins and nucleic acids of suspensions of thymic and lymphosarcoma cells. Evidence of actual synthesis of new protein by lymphocytes under the experimental conditions studied was derived from a variety of data. The reincubation experiments described (Figs. 2 and 5) indicate that incorporation of isotopic amino acids into cellular protein was essentially irreversible, and it would seem unlikely that a few amino acid residues in the cellular protein were in constant amino acid exchange with the medium. The fact that specific activities of protein samples remained unchanged after treatment with reagents such as mercaptoethanol and performic acid suggests that the radioactivity of these protein samples was not caused by small molecules, *e.g.* glutathione, biosynthesized from glycine-2-C^{14} and bound to protein by means of disulfide linkages. More conclusive evidence for actual incorporation of glycine-2-C^{14} into newly synthesized proteins was provided by isolation from radioactive peptides, obtained from labeled lymphocyte protein, of glycine and serine as the only radioactive amino acids in these peptides.

Prior adrenalectomy or the presence of a lymphosarcoma in the rat decreased significantly the rate of incorporation of glycine into thymic cellular proteins and nucleic acids (Table I). These data paralleled the decline observed in the respiratory rates of such cells. The rates of incorporation of radioactivity into proteins and nucleic acids of both thymic and lymphosarcoma cells

were stimulated by the simultaneous oxidation of the substrate, *e.g.* glucose (Table II). The findings are consonant with the energy-requiring nature of protein and nucleic acid syntheses and with the correlation between the steroids which most effectively inhibit respiration and glucose oxidation (5) by lymphocytes and those which retard incorporation of glycine into the proteins and nucleic acids of these cells. Lymphosarcoma cells, in contrast to thymic lymphocytes, incorporated significant radioactivity from glycine-2-C^{14} into proteins and nucleic acids under anaerobic conditions, provided glucose was added to the incubation medium. Thus the dependence of this tumor upon anaerobic glycolysis for energy production was indicated (Tables I and II).

The steroids studied differ in their effectiveness in retarding the incorporation phenomena studied (Table III). The potency of a steroid in this regard was directly related to its activity in producing inhibition of glucose oxidation and respiration of lymphocytes. Thus, of the compounds tested, 17α-ethyl,19-nortestosterone was the most effective; it was inhibitory in concentrations of 10^{-5} M (*cf.* (5)). It may be noted that, in this respect, this steroid is a more potent inhibitor of the metabolism of lymphocytes than are other well known metabolic poisons, *e.g.* cyanide. Kit *et al.* reported that 100 μg. of cortisone or hydrocortisone per ml. of incubation medium produced approximately 37 per cent inhibition of incorporation of glycine-1-C^{14} into the total protein (8), and an inhibition of incorporation of radioactive phosphate into the nucleic acids (25) of rat thymic cells.

The finding that 17α-ethyl,19-nortestosterone, of the steroids studied, was the most potent inhibitor of glycine-2-C^{14} incorporation into proteins and nucleic acids of lymphocytes was unexpected. It is true that this steroid is a very effective inhibitor of lymphocyte respiration and glucose oxidation when added to these cells *in vitro* or in lymphocytes obtained 4 hours after steroid administration (5). Nevertheless, this steroid apparently has no gross lymphocytolytic activity either *in vivo* or *in vitro*, and, indeed, exhibits an anabolic activity when administered to experimental animals (26) and to man (27, 28). The inhibitory effects of 17α-ethyl,19-nortestosterone on lymphocyte metabolism either may be unique for this histological unit or may reflect an acute type of action of this steroid. This point warrants further study. In any event, steroids known to be highly potent in their lymphocytolytic actions, *e.g.* hydrocortisone, were less potent inhibitors of the phenomena studied in these experiments than were 17α-ethyl,19-nortestosterone and other steroids known not to involute lymphoid tissue, *e.g.* deoxycorticosterone and 17α-methyltestosterone. Indeed, administration of 17α-ethyl,19-nortestosterone or deoxycorticosterone to normal or adrenalectomized rats yielded thymic lymphocytes which exhibited degrees of inhibition of glycine incorporation similar to those found when either steroid was added *in vitro*. In such rats, there was no evidence of thymic involution. On the other hand, use of hydrocortisone under the same experimental conditions produced the well known decrease in thymic weight; the cells of such tissue behaved *in vitro* in a manner similar to those from tissue of rats given injections of steroids which did not alter thymic weight. Thus it is not at present apparent what relation, if any, exists between the lymphocytolysis produced by certain steroids and the capacity of the latter to inhibit energy-producing and synthetic mechanisms in lymphocytes. It may be noted that certain nonsteroidal reagents, *e.g.* methylene blue and 2,4-dinitrophenol, known to uncouple oxidative phosphorylation, were also capable of inhibiting incorporation of glycine-2-C^{14} into proteins and nucleic acids of thymic lymphocytes in concentrations which did not inhibit oxygen uptake (Table VI). There is no reported effect of these agents on lymphocyte morphology. Moreover, the dubiousness of the dependence of the steroid effect on its inhibition of respiration is further indicated by the ability of several steroids to inhibit glycine incorporation into lymphosarcoma cells under anaerobic conditions (Table VIII). Rather the data indicate that interference with energy production, either aerobically or anaerobically, is the prime factor in influencing the rate of glycine-2-C^{14} incorporation.

The data of Fig. 5 do not exclude the possibility that inhibitions by steroids of the incorporation of glycine-2-C^{14} may actually be the result of dilution of radioactive glycine by nonlabeled glycine made available from lymphocyte protein under the catabolic influence of steroid. However, evidence contraindicative of such a possibility was provided by experiments (Table IV) in which lymphocytes were labeled *in vivo*; the radioactivity of proteins and nucleic acids of these cells was not diminished by subsequent isolation and incubation of the cells with 17α-ethyl,-19-nortestosterone.

Preincubation of lymphocytes with steroid, and subsequent study of these cells after steroid removal indicated that, whatever the basis of the inhibitory effects of the compound, they were not irreversible (Table V). This might imply that the steroid did not exert its influence by alteration of the permeability of the cells. Support for this view is derived from evidence that the inhibitory effects of certain steroids, *e.g.* deoxycorticosterone, on respiration and glucose oxidation *in vitro* have been observed with cell-free extracts of thymic[11] and lymphosarcoma cells[8]. The data seem to preclude a significant role for the steroids in such processes as amino acid transfer (29) into lymphocytes. Koritz and Dorfman (30) found that deoxycorticosterone added *in vitro* to reticulocytes inhibited both oxygen consumption and incorporation of glycine-1-C^{14} into the total proteins of these cells. Preincubation with the steroid had no effect on the incorporation rate up to a 2-hour preincubation period; evidence was presented which indicated that deoxycorticosterone had no influence on the permeability of the cells to glycine or potassium ion.

A possible relationship between steroid structure and the effectiveness of a compound in inhibiting oxygen consumption, glucose oxidation (5), and incorporation of glycine-2-C^{14} into proteins and nucleic acids of lymphocytes seems to obtain. The possible significance of this relationship and its basis await further studies in progress with cell-free preparations.

SUMMARY

1. Lymphocyte suspensions prepared from rat thymus or Murphy-Sturm lymphosarcoma tissue incorporate glycine-2-C^{14} into the total proteins and nucleic acids of these cells. Evidence that synthesis of protein and nucleic acids has occurred under the experimental conditions which obtained is presented.

2. Under anaerobiosis the synthetic reactions of lymphosarcoma cells were markedly dependent upon the presence of glucose; incorporation by thymic lymphocytes was dependent upon aerobiosis.

3. A variety of steroids, added *in vitro* to normal and malignant lymphocyte suspensions in concentrations of the order of

[11] L. A. Jedeikin and A. White, unpublished observations.

10^{-5} M, inhibited, in a generally parallel manner, respiration and glycine-2-C^{14} incorporation into proteins and nucleic acids. The inhibitory influence of the steroids was also observed under conditions of anaerobiosis with normal and malignant lymphocytes.

4. Injection of a steroid into rats at 24 and 4 hours before decapitation yielded lymphocytes which, when studied *in vitro*, exhibited degrees of inhibition of respiration and glycine-2-C^{14} incorporation similar to those observed when the steroids were added *in vitro*.

5. Incubation of lymphocytes with steroid did not produce irreversible alterations in the ability of lymphocytes to respire and to incorporate glycine-2-C^{14}.

6. It is suggested that certain steroids interfere with the aerobic or anaerobic production of energy by normal and malignant lymphocytes and that this is reflected in a diminished incorporation of radioactive amino acids into the proteins and nucleic acids of these cells.

Acknowledgment—Miss Bernyce Dvorkin provided valuable assistance in the experiments described in this paper.

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O-Phosphoserine Phosphatase from Baker's Yeast

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(Received for publication, July 3, 1958)

The formation of phosphoserine as an intermediate in the synthesis of serine from 3-phosphoglyceric acid has been demonstrated by Ichihara and Greenberg (1). A dephosphorylation of phosphoserine was proposed as a step in the reaction sequence and was ascribed to nonspecific phosphatases present in the rat liver preparations studied.

The present communication describes the isolation and the properties of a specific phosphoserine phosphatase obtained from baker's yeast. The partially purified enzyme can be used as a specific reagent for the determination of phosphoserine. A similar enzyme was partially purified from extracts of *Alcaligenes faecalis*.

While this work was in progress, Borkenhagen and Kennedy (2) and Neuhaus and Byrne (3) reported on phosphoserine phosphatase obtained from rat liver and from chicken liver, respectively.

EXPERIMENTAL

Analytical Methods—Inorganic phosphate was determined according to Fiske and SubbaRow (4) and by the method of Lowry and Lopez (5). Ammonia was assayed with Nessler's reagent. Protein was measured turbidimetrically (6).

Materials—DL-Phosphoserine and phosphoethanolamine were obtained from Mann Research Laboratories, Inc., New York. L-Phosphoserine and D-phosphoserine were kindly donated by Dr. F. C. Neuhaus. Phosphovitin was a gift from Dr. M. Rabinowitz. 3-Phosphohydroxypyruvate was generously donated by Dr. C. E. Ballou. Alumina Cγ was a commercial preparation of the Sigma Chemical Company, St. Louis, Missouri. Cellulose DEAE Type 20 ion exchange agent (7) was a product of the Brown Company, Berlin, New Hampshire. Baker's yeast was obtained from The Fleischmann Company, Mount Vernon, New York. The yeast was dried overnight under a stream of air at room temperature and was then stored at 2°.

Assay of Phosphoserine Phosphatase—The enzymatic activity was measured in a system containing 10 μmoles of DL-phosphoserine, 130 μmoles of Tris[1] buffer, pH 7.4, 10 μmoles of $MgCl_2$, and enzyme in a final volume of 2 ml. Incubation was carried out for 20 minutes at 30°. The reaction was stopped by the addition of 1 ml. of 15 per cent trichloroacetic acid. 1 unit was defined as that amount of enzyme that catalyzes the formation of 1 μmole Pi in 20 minutes. The amount of Pi formed was found to be proportional to the amount of enzyme in the range from 0.6 to 2.4 units. With 1.5 units of enzyme the amount of Pi formed was proportional to the reaction time in the range from 0 to 40 minutes.

Isolation of Enzyme

Step 1. Preparation of Crude Yeast Extract—300 gm. of dried baker's yeast were suspended in 900 ml. of 0.1 M sodium bicarbonate and incubated at 41° for 2.5 hours with occasional shaking. The suspension was centrifuged and the supernatant solution was cooled to 2°. The precipitate was re-extracted with 600 ml. of bicarbonate for 2.5 hours at 41°. After centrifugation the precipitate was discarded, and the supernatant solutions of the first and second extractions were combined (1030 ml.). All subsequent operations were carried out at 0–2°. 50 ml. of a 3 per cent solution of protamine sulfate, adjusted to pH 6.5, were added with stirring to the crude yeast extract. After standing for 5 minutes the solution was centrifuged, and the small amount of precipitate was discarded.

Step 2. Fractionation with Ammonium Sulfate—Throughout the purification procedure, fractionation with ammonium sulfate was carried out at a pH from 6 to 7. The pH was maintained by addition of 2 N ammonia. 41.7 gm. of ammonium sulfate were added for each 100 ml. of the yeast extract. The precipitate was removed by centrifugation at 10,000 $\times$ g for 10 minutes. An additional 15.2 gm. of ammonium sulfate were added per 100 ml. of the supernatant solution, and stirring was continued for 20 minutes after the ammonium sulfate had dissolved. The precipitate obtained after centrifugation at 16,000 $\times$ g for 30 minutes was taken up in a minimal amount of water and dialyzed overnight against 4 l. of 0.002 M Tris buffer, pH 7.7, containing 0.001 M $MgCl_2$.

Step 3. Removal of Inactive Protein by Cγ—607 mg. of Cγ[2] in 4.4 ml. were added to the dialyzed protein solution (46 ml. with 24.4 mg. of protein per ml.). The suspension was centrifuged at 6000 $\times$ g for 5 minutes and the precipitate was discarded.

Step 4. Fractionation on Cellulose DEAE Column—The supernatant solution (50 ml. with 1000 mg. of protein) was applied to a cellulose DEAE column (7) (5 cm. $\times$ 7 cm.^{2}) which had been washed with 150 ml. of 0.005 M Tris buffer, pH 7.7, containing 0.001 M $MgCl_2$. The flow rate through the column averaged 3 ml. per minute. Protein was eluted from the column with solutions containing 0.05 M Tris, pH 7.7, 0.001 M $MgCl_2$, and the following different concentrations of KCl: Solution I, no KCl; II, 0.04 M KCl; III, 0.1 M KCl; IV, 0.2 M KCl; and V, 0.4 M KCl. 40 ml. portions of each solution were successively applied to the column. The collection of eluate, in

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[1] The abbreviations used are: Tris, tris(hydroxymethyl)aminomethane; Pi, inorganic phosphate.

[2] The exact amount of Cγ required to remove the maximal amount of inactive protein without adsorbing the enzyme was determined on small samples.

TABLE I

Purification of phosphoserine phosphatase

Step	Protein concentration	Total units	Specific activity	Recovery
	mg./ml.		*units/mg. protein*	%
Crude yeast extract	8.4	10,400	1.2	100
Ammonium sulfate precipitate (68–90%)	24.4	4,150	3.7	40
Cγ	20.0	3,950	3.9	38
Cellulose column and precipitation by ammonium sulfate	11.7	2,160	33.6	21

fractions of 10 ml., was started when Solution III was applied to the column, because the enzyme is eluted by Solutions III and IV. Enzyme of high specific activity was recovered in 5 to 6 fractions which contained 70 to 80 per cent of the total activity. These fractions were pooled (50 ml.), and the protein was precipitated by addition of 30.5 gm. of ammonium sulfate (61 gm. per 100 ml. of the protein solution). The precipitate obtained after centrifugation at 12,000 × *g* for 25 minutes was drained thoroughly and taken up in 3 to 4 ml. of 0.02 M Tris buffer, pH 7.4, which contained 0.001 M $MgCl_2$. When stored at −20° this enzyme preparation showed no loss of activity for at least 1 month. A summary of the purification and recovery of enzyme is given in Table I.

RESULTS AND DISCUSSION

Properties of Phosphoserine Phosphatase

The reaction of the purified enzyme preparation on phosphoserine seems to be limited to the direct hydrolysis of the latter to Pi and serine. The following observations support this conclusion. No ammonia was formed during the reaction. The amount of Pi measured was the same whether assayed with the strongly acid reagent of Fiske and SubbaRow or with the reagent of Lowry and Lopez at pH 4.5. Paper chromatograms

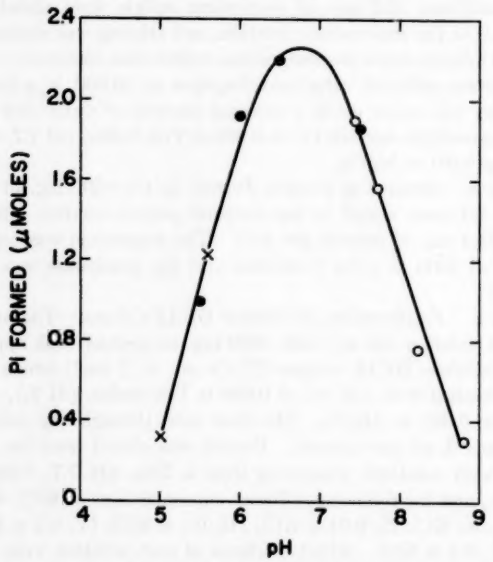

FIG. 1. Rate of dephosphorylation of phosphoserine as a function of pH. The assay system described in the text was employed except that the buffers were: acetate (×——×), histidine (●——●) and Tris (○——○) at a final concentration of 0.05 M.

TABLE II

Specificity of cation requirement for activity of phosphoserine phosphatase

The reaction mixture contained in a final volume of 2 ml.: 10 μmoles of DL-phosphoserine; 140 μmoles of Tris buffer, pH 7.4; 5 μmoles of a given salt as indicated below; and 2 units of an enzyme preparation which had been dialyzed overnight against 0.002 M Tris buffer, pH 7.4. Incubation was for 20 minutes at 30°.

Salt added	Pi formed
	μmoles
None	0.0
$MgCl_2$	1.6
$CoCl_2$	1.2
$Co(NO_3)_2$	1.2
$FeCl_2$	0.7
$NiCl_2$	0.5
$ZnCl_2$	0.1
$MnCl_2$	0.0
$CaCl_2$	0.0
$BaCl_2$	0.0
$CuCl_2$	0.0
$CdSO_4$	0.0

TABLE III

Inhibitors of phosphoserine phosphatase

Inhibitors were tested in the enzyme assay system described in the text.

Inhibitor tested	Concentration	Inhibition
	mole/l.	%
NaF	0.010	100
$MnCl_2$	0.002	86
$CaCl_2$	0.005	90
L-Serine	0.001	8
L-Serine	0.003	19
L-Serine	0.005	29
L-Serine	0.010	43
L-Serine	0.020	62

TABLE IV

Quantitative dephosphorylation of L-phosphoserine

The reaction mixtures contained in a final volume of 2 ml.: 10 μmoles of $MgCl_2$; 130 μmoles of Tris buffer, pH 7.4; 6 units of enzyme; and substrate as indicated. Incubation was for 30 minutes at 30°.

L-Phosphoserine	Compound added	Pi formed
μmoles	*μmoles*	*μmoles*
1		1.03
2		1.96
1	L-Serine, 20	0.98
1	D-Phosphoserine, 1	1.18
1	D-Phosphoserine, 10	1.87
1	Adenosine triphosphate, 10	1.05
1	DL-Phosphothreonine, 10	1.03

of the reaction mixture, developed with phenol containing 3 per cent ammonia (8), revealed two ninhydrin-positive spots corresponding in their mobility to phosphoserine and serine. Neither alanine nor glycine was detected.

pH Optimum and Affinity for Substrate—The optimum pH for phosphoserine phosphatase activity is from 6.5 to 7.0 (Fig. 1). At pH 5 and 8.8 the activity is 15 per cent of what it is when the pH is 6.5. When the pH is 7.4 the K_m for L-phosphoserine is 4×10^{-4} and for D-phosphoserine it is 8×10^{-3}.

Substrate Specificity—L-Phosphoserine was dephosphorylated 6 times faster than D-phosphoserine when tested at a concentration of 0.005 M because of the lower affinity of the latter for the enzyme. At a concentration of 0.04 M both were dephosphorylated at the same rate.

The following compounds, tested at a concentration of 0.005 M, did not yield measurable amounts of Pi when incubated with 4 units of enzyme in 0.065 M Tris buffer, pH 7.4, for 20 minutes at 30°: DL-phosphothreonine; *o*-phosphoethanolamine; 3-phosphohydroxypyruvate; adenosine tri-, di-, and monophosphate; fructose-1,6-diphosphate; sedoheptulose-1,7-diphosphate; glucose-6-phosphate; ribose-5-phosphate; and 3-phosphoglyceric acid. Phosphovitin and casein, which were tested at a concentration of 2.5 mg. per ml., also failed to yield Pi. The purified enzyme preparation was, however, still contaminated with inorganic pyrophosphatase. The ratio of inorganic pyrophosphatase activity to phosphoserine phosphatase activity decreased from 80:1 in the crude yeast extract to 0.5:1 in the purified preparation. When cobalt was substituted for magnesium in the assay system (Table II), the ratio could be further decreased to 0.05:1.

Requirement for Divalent Cation—Dialyzed preparations of the enzyme were inactive in the absence of a divalent cation. Although magnesium was required for maximal activity, other divalent cations could also activate the enzyme but not as efficiently (Table II). It is of interest to note that the yeast enzyme survived dialysis against Tris buffer in the absence of magnesium whereas a similar enzyme, obtained from *Alcaligenes faecalis*, was found to be irreversibly inactivated unless magnesium was added to the dialysis medium.

Inhibitors—Fluoride, manganese, and calcium salts were shown to be effective inhibitors of phosphoserine phosphatase, while L-serine caused appreciable inhibition only at relatively high concentrations (Table III). It should be noted that the phosphoserine phosphatase obtained from rat liver (2) and from chicken liver (3) is strongly inhibited in the presence of low concentrations of L-serine. No inhibition (less than 10 per cent) could be detected in the presence of DL-phosphothreonine, phosphoethanolamine, DL-threonine and L-cysteine when tested at a concentration of 0.01 M.

Quantitative Dephosphorylation of Phosphoserine

In view of the high affinity and specificity of phosphoserine phosphatase for L-phosphoserine, the enzyme may be useful as a reagent for the specific determination of the latter. As shown in Table IV, limiting amounts of L-phosphoserine can be dephosphorylated quantitatively within a relatively short time. Mixtures of L- and D-phosphoserine were not dephosphorylated quantitatively under these conditions because of the low affinity of the enzyme for the latter. The presence of a great excess of L-serine, adenosine triphosphate, or DL-phosphothreonine did not interfere with the quantitative dephosphorylation of L-phosphoserine.

SUMMARY

A specific phosphoserine phosphatase has been isolated and partially purified from dried baker's yeast. The following compounds were not dephosphorylated by the purified enzyme: DL-phosphothreonine; *o*-phosphoethanolamine; phosphohydroxypyruvate; adenosine tri-, di-, and monophosphate; fructose-6-phosphate; fructose-1,6-diphosphate; sedoheptulose-1,7-diphosphate; glucose-6-phosphate; ribose-5-phosphate; 3-phosphoglyceric acid; phosphovitin; and casein. The enzyme requires a divalent cation for activity. Maximal rates are obtained in the presence of magnesium ions. The K_m for L-phosphoserine is 4×10^{-4}, and the optimal pH for the reaction is from 6.5 to 7.0. Fluoride, manganese, and calcium ions are potent inhibitors of the reaction. The application of the enzyme to the specific determination of L-phosphoserine is outlined.

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Effects of Vitamin E Deficiency on Tissue and Erythrocyte Glutathione

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(Received for publication, July 11, 1958)

Several laboratories have reported observations which point to the existence of metabolic interrelationships between vitamin E and the sulfur-containing amino acids. The work of Schwarz and of Chernick *et al.* (1, 2) has shown that either vitamin E or cystine alone affords protection against the necrotic liver degeneration produced in various animals by diets deficient in the sulfur-containing amino acids and vitamin E. Olson *et al.* (3) made similar observations and in addition found the rate of incorporation of S^{35}-cystine into liver coenzyme A to be depressed in vitamin E-deficient rats. Tallan (4) noted elevated GSH levels in muscle extracts from vitamin E-deficient rabbits.

The present work was undertaken to study further the interrelationships of GSH and vitamin E. It was found that the GSH content of liver, as well as that of skeletal muscle, was increased in vitamin E-deficient rabbits. Erythrocyte GSH was also increased but to a lesser extent. Labeled glycine was incorporated into the GSH of the muscles at an increased rate.

EXPERIMENTAL

Male New Zealand rabbits, which weighed approximately 1000 gm., were placed in groups. In the first experiment there were three groups. One received the purified vitamin E-deficient diet used by Young and Dinning (5), the second group received the same diet supplemented orally with 4 mg. of α-tocopheryl acetate per kg. of body weight twice weekly, and the third group was given a stock diet of Pillsbury rabbit pellets. As soon as a deficient rabbit exhibited signs of muscular paralysis, blood was withdrawn by heart puncture and the animal was killed. Samples of liver and skeletal muscle were immediately taken and frozen with solid carbon dioxide. At the same time, blood and tissue samples were obtained from vitamin E-supplemented and stock animals.

GSH was determined in metaphosphoric acid filtrates by the alloxan method of Patterson and Lazarow (6). This procedure makes use of the ability of alloxan to react with GSH to form a compound with a maximal absorption at 305 mμ. Since the color development is sensitive to changes in pH, the reaction mixture was adjusted to give a final pH which was close to 7.5, as recommended by Ball *et al.* (7). Optical densities were determined in a Beckman model DU spectrophotometer.

Inasmuch as the alloxan method has not been used extensively for the determination of tissue GSH, all liver and muscle samples were also analyzed for GSH by the nitroprusside method of Grunert and Phillips (8). As a further check, the total nonprotein sulfhydryl content of many of the tissue samples was determined by amperometric titration (9).

Hematocrit values were obtained for the blood samples by means of Wintrobe tubes. The concentration of GSH in the erythrocytes was calculated from the hematocrit value and the concentration in whole blood.

For the experiment in which the incorporation of labeled glycine into muscle GSH was studied, vitamin E-deficient and vitamin E-supplemented rabbits were given 15 μc. of glycine-2-C^{14} (Tracerlab, Inc.) per kg. of body weight in isotonic saline by intravenous injection. Since it had been shown that the incorporation of labeled glycine into muscle GSH approaches a plateau approximately 4 hours after injection (10), the rabbits were killed and samples of skeletal muscle were taken for analysis at 4 hours. GSH was isolated as the copper mercaptide from weighed portions of the frozen tissue (11). Suspensions of the mercaptide were deposited for counting on aluminum disks by means of the plating procedure of Hutchens *et al.* (12). The counting of the GSH copper mercaptide was carried out in a gas flow counter as described earlier (13).

Since there have been conflicting reports concerning the effect of vitamin E deficiency on the concentration of free glycine in muscle (4, 14, 15), the concentration of this amino acid in the various tissues of vitamin E-deficient and vitamin E-supplemented rabbits was measured. In order to make the determination, a protein-free extract was obtained by homogenization of 1 part of a tissue sample with 3 parts of distilled water after which 1 part of 25 per cent metaphosphoric acid was added. After the mixture was centrifuged and filtered, DNP[1]-derivatives of the amino acids present in the filtrate were prepared by an adaptation of the method of Sanger (16) as modified by Krol (17). The derivatives were chromatographed on a 9 × 60-mm. silicic acid column (18) with the use of a developer which consisted of 2 per cent acetic acid and 10 per cent acetone in ligroin (19). The bands which appeared above the DNP-glycine band were removed, and the DNP-glycine was eluted with 3.5 ml. of acetone. After dilution of the eluate to 10 ml. with ethanol, the optical density was measured at 345 mμ. Corrections for losses were determined by the use of known amounts of glycine. Each analysis was carried out in duplicate.

RESULTS AND DISCUSSION

The results of the first experiment are given in Table I. It will be noted that the liver GSH of the supplemented rabbits increased progressively with the length of time required for the development of muscular paralysis. In each case, however, the level found in the deficient animal was higher than that observed in the corresponding supplemented animal. The increase

[1] The abbreviation used is: DNP, 2,4-dinitrophenyl.

TABLE I

GSH levels of vitamin E-deficient and control rabbits

Rabbit No.	Diet	Duration of diet*	Liver GSH		Muscle GSH		Erythrocyte GSH
			Alloxan method	Nitroprusside method	Alloxan method	Nitroprusside method	Alloxan method
		days	*μmoles/100 gm.*	*μmoles/100 gm.*	*μmoles/100 gm.*	*μmoles/100 gm.*	*μmoles/100ml.*
1	Deficient	19	1360	1340	237	238	506
2	Supplemented	19	315	300	153	146	349
3	Deficient	26	1480	1740	226	215	557
4	Supplemented	26	301	384	175	138	432
5	Deficient	31	1310	1210	281	264	707
6	Supplemented	31	690	645	151	110	586
7	Deficient	32	1640	1670	255	227	587
8	Supplemented	32	984	987	173	130	435
9–12†	Stock	26–32	1020	1010	169	130	426

* Time required for the development of muscular paralysis in the deficient animals.

† Values for the stock rabbits are the averages of four animals.

TABLE II

Incorporation of C^{14} of glycine-2-C^{14} into GSH of skeletal muscle by vitamin E-deficient and vitamin E-supplemented rabbits

Rabbit No.	Diet	c.p.m./mg. of muscle GSH	c.p.m. in GSH/gm. of muscle
1	Deficient	566	393
2	Supplemented	372	179
3	Deficient	418	323
4	Supplemented	266	144
5	Deficient	500	384
6	Supplemented	310	165

TABLE III

Concentration of free glycine in tissues of vitamin E-deficient and vitamin E-supplemented rabbits

The results are in milligrams per 100 gm., wet weight. There were four rabbits in each group.

Tissue	Vitamin E-deficient	Vitamin E-supplemented
Liver	28 ± 1.7*	26 ± 2.8
Muscle	36 ± 2.1	41 ± 6.3
Whole blood	5.0 ± 0.3	5.5 ± 1.7

* Standard deviation of the mean.

ranged from approximately 300 per cent for the first animals to be killed to approximately 70 per cent for the last ones. Muscle and erythrocyte GSH levels were also higher in the deficient animals. The average increase in the muscles was 54 per cent (alloxan method) and in the erythrocytes, 33 per cent. The muscles and erythrocytes of the supplemented animals did not show the progressive rise in GSH content which was observed in the livers. As indicated in Table I, the liver GSH levels of the rabbits on the stock diet were higher than those of the animals given the purified vitamin E-deficient diet supplemented with α-tocopherol, but in no instance were they as high as the levels of the deficient animals. The effects on muscle GSH levels are in agreement with the results obtained by Tallan (4).

No explanation is at hand for the higher liver GSH values obtained for the rabbits on the stock diet as compared with those on the supplemented purified diet. The diets contained comparable amounts of the sulfur-containing amino acids. This finding is in accord, however, with previous experience in this laboratory where animals maintained on purified diets have been found to have uniformly lower liver GSH levels than similar animals fed a stock diet.

In the second experiment, it was found that vitamin E-deficient rabbits incorporated more of the C^{14} of labeled glycine into muscle GSH than did the supplemented animals (Table II). This observation suggests that the turnover rate of muscle GSH is accelerated by vitamin E deficiency. Such an interpretation, however, would not be valid if the size of the free glycine pool were greatly decreased in vitamin E-deficient animals. Such a decrease would obviously result in greater radioactivity in the GSH through an increase in the specific activity of the free amino acid. The data in Table III indicate that the vitamin E deficiency did not significantly alter the concentration of glycine in the tissues studied. These results are in substantial agreement with those reported by Dinning *et al.* (14), who worked with the same diets used in the present work but a different method of analysis.

As a result of these experiments it seems that the metabolism of GSH in the tissues of the rabbit is, to some degree at least, controlled by vitamin E.

SUMMARY

The effects of vitamin E deficiency on liver, skeletal muscle, and erythrocyte glutathione levels have been investigated in rabbits. Deficiency of the vitamin produces increases in the glutathione content of these tissues and the erythrocytes. These changes are greatest in the liver and least marked in the erythrocytes. The incorporation of the C^{14} of labeled glycine was enhanced by vitamin E deficiency. These results suggest a role of vitamin E in the regulation of glutathione metabolism.

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The Enzymatic Cleavage of Canavanine to Homoserine and Hydroxyguanidine*

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(Received for publication, July 14, 1958)

Previous studies have shown canavanine to be a potent antagonist of arginine in *Neurospora* (1) and in some bacteria, but not in others (2, 3). *Streptococcus faecalis* belongs to the resistant group of bacteria and has been shown to degrade canavanine in two distinct ways: by reductive cleavage to guanidine and homoserine (4) and, to a lesser extent, by hydrolytic cleavage to ammonia and O-ureidohomoserine (5).

In an extension of these studies, a pseudomonad capable of utilizing canavanine as a sole source of carbon and nitrogen was isolated from soil by the enrichment culture technique. The initial attack on canavanine by this organism was found to consist in a hitherto undescribed cleavage of this amino acid to yield hydroxyguanidine and homoserine. The present paper deals with the demonstration of this reaction.

EXPERIMENTAL

Culture Medium—The medium used both for isolation and subsequent culture of the organism contained per l. 2.0 gm. of L-canavanine, 1.0 gm. KH_2PO_4, 0.5 gm. of $MgSO_4 \cdot 7H_2O$, 3.0 mg. of $CaCl_2$ and 3.0 mg. of $FeCl_3 \cdot 6H_2O$. It was adjusted to pH 7.0 with potassium hydroxide and sterilized by autoclaving at 15 pounds of pressure for 10 minutes. Canavanine is somewhat labile to autoclaving. It was sterilized by filtration and added after the remainder of the medium was autoclaved. Stock cultures of the isolated organism were maintained on slants of the same medium solidified with 2 per cent of purified agar.

Isolation of Organism[1]—Erlenmeyer flasks (50 ml.) containing 10 ml. of the basal medium were inoculated with 100 to 500 mg. of mud from San Francisco Bay and incubated without shaking at 30°. After 48 hours, when abundant turbidity had developed, 0.01 ml. aliquots were transferred to fresh medium and incubation was continued in the same way. After three such successive transfers, the culture was streaked onto solid basal medium. The single predominating colony type was replated, and an isolated colony picked for further study. The organism forms a yellow-green fluorescent pigment, and from its microscopic appearance and cultural characteristics appears to be a species of the genus *Pseudomonas*.

Quantitative Methods—Homoserine and canavanine were estimated by the quantitative paper chromatographic method of Giri *et al.* (6). Canavanine also was estimated by a slightly modified Archibald procedure described previously (4). Hydroxyguanidine was estimated by the method of Walker (7). Homoserine, guanidine, and ammonia were found not to interfere in the determination of hydroxyguanidine. Canavanine, however, gave a color value equivalent to 13 per cent of that of hydroxyguanidine on the molar basis; accordingly, the value found for hydroxyguanidine was corrected for the amount of unchanged canavanine present.

Synthesis of Hydroxyguanidine Hydrochloride—The reaction originally described by Prätorius-Seidler (8) and recently utilized by Adams *et al.* (9) was used for preparing hydroxyguanidine. Since the isolation of hydroxyguanidine in pure form has not been previously described, its method of preparation is given in detail below. Cyanamide (2.1 gm., 0.05 mole) was dissolved in 4 ml. of water and 3.5 gm. (0.05 mole) of hydroxylamine hydrochloride were added. After standing at room temperature for 4 to 11 days, the reaction mixture was placed on a Dowex 50 (H^+) column (2.3 × 50 cm., 200 to 400 mesh) and the column first was washed with about 900 ml. of water and then developed with 1 N hydrochloric acid. The hydroxyguanidine was detected in the fractions collected by its brown color reaction with alkaline ferricyanide-nitroprusside reagent (10), and was eluted from the column in the fraction between 1.7 and 2.3 l. of the acid effluent. These fractions were concentrated in a vacuum to a syrup which was dried further over sodium hydroxide pellets in a vacuum desiccator. The resulting white crystalline solid was recrystallized by dissolving it in 5 ml. of absolute ethanol and slowly adding 50 ml. of ether to the solution; the yield was 0.75 to 0.86 gm. (13 to 16 per cent). The product is quite hygroscopic and melts at 88–90° (corrected)

$CH_5N_3O \cdot HCl$

Calculated: C 10.77, H 5.42, N 37.67

Found: C 10.47, H 5.19, N 37.34

The N-2,4-dinitrophenyl derivative of hydroxyguanidine was prepared according to the method of Sanger (11). The derivative was purified by washing it with a small amount of ether, in which it is only slightly soluble, and recrystallizing it from ethanol. It melted at 199 to 200° (corrected) with decomposition.

$C_7H_7N_5O_5$

Calculated: C 34.86, H 2.93, N 29.04

Found: C 35.11, H 3.12, N 29.21

* Supported in part by Grant E-1575 from the United States Public Health Service.

[1] We are indebted to Dr. B. E. Volcani for assistance in this phase of the work.

RESULTS

Degradation of Canavanine by Growing Cultures—After various times of incubation in the described medium, the cultures were centrifuged and the supernatant solutions concentrated in a vacuum and examined by paper chromatography. After only 2 hours of growth, the medium showed traces of homoserine and an unknown substance which did not react with ninhydrin but gave a characteristic green color with α-naphthol-diacetyl spray (12). The amounts of both substances increased during the first few hours of incubation; thereafter, homoserine decreased while the unknown continued to increase in amount. The homoserine was identified by paper chromatographic comparisons with authentic homoserine in pyridine-water (80:20), in 77 per cent ethanol, and in *n*-butanol-acetic acid-water (40:10:50). The identification was confirmed by eluting the substance from a paper chromatogram, heating the eluate with dilute hydrochloric acid, and chromatographing the resulting solution on paper. In addition to homoserine, a compound was formed that gave the same yellowish-brown color with ninhydrin as homoserine lactone and also migrated on paper identically with an authentic sample of homoserine lactone. The unknown substance was identified as hydroxyguanidine by paper chromatographic comparisons with synthetic hydroxyguanidine in absolute ethanol-water-acetic acid (77:23:1) (R_F, 0.59), in absolute ethanol-1 M ammonium hydroxide (77:23) (R_F, 0.58), and in *n*-butanol-acetic acid-water (4:1:1) (R_F, 0.23). Both natural and synthetic samples gave the same green color when the chromatograms were sprayed with α-naphthol-diacetyl reagent, and the same purple color when sprayed with alkaline ferricyanide-nitroprusside reagent. The green color formed with the α-naphthol-diacetyl reagent seems to be quite characteristic of hydroxyguanidine since both canavanine and guanidine give a pink color. The identity with hydroxyguanidine of the unknown substance was further confirmed by its isolation, as described later.

After 18 hours of incubation, both canavanine and homoserine had disappeared from the medium. The concentration of hydroxyguanidine increased until all canavanine had disappeared, then remained constant even after 30 hours of incubation, showing that it was not further metabolized by the organism. It was found that the test organism grew equally as well when homoserine, arginine, citrulline, ornithine, alanine, or serine was substituted for canavanine as the sole source of carbon and nitrogen, but did not grow when hydroxyguanidine was substituted. The cleavage reaction apparently serves the organism solely as a means for formation of homoserine, which serves as the actual substrate for growth.

Isolation of Hydroxyguanidine—A culture grown in 500 ml. of medium was centrifuged after 12 hours of incubation and the supernatant liquid concentrated in a vacuum to 25 ml. An equal volume of ethanol was added, the precipitated salts were filtered, the filtrate was further concentrated, and the concentrate placed on a Dowex 50 (H^+) column (2.6 × 45 cm., 200 to 400 mesh). The column was washed with 1 l. of water, then developed with 1 N hydrochloric acid. The compound giving the green color with α-naphthol-diacetyl spray was obtained in the 2000 to 2500 ml. fraction of the acid effluent from the column. This fraction was concentrated to dryness in a vacuum and the residue recrystallized by dissolving it in 2 ml. of ethanol and slowly adding 20 ml. of ether. An 11 mg. sample of the slightly impure product yielded 25 mg. of crude 2,4-dinitrophenyl derivative which, on recrystallization from ethanol, gave 14 mg. of pure product melting at 198–199° (corrected) with decomposition, and which showed no depression in decomposition point when mixed with authentic dinitrophenylhydroxyguanidine.

Degradation of Canavanine by Cell-Free Extracts—Cells from an 18 hour culture were collected by centrifuging and washed twice with distilled water. The washed cells were suspended in 0.02 M phosphate buffer, pH 7.2 (18.5 mg. dry weight of cells per ml.), and broken by treating them for 20 minutes in a Raytheon 9 kc sonic oscillator. The resulting opalescent solution was centrifuged, and the supernatant solution dialyzed against deionized water in the cold room. The dialyzed preparation degraded canavanine to homoserine and hydroxyguanidine with an optimal pH near 7 (Table I). Homoserine and hydroxyguanidine are formed in equimolar amounts (Table I). The over-all reaction thus corresponds to a simple hydrolytic cleavage of canavanine.

TABLE I

Stoichiometry and pH effects on degradation of canavanine by cell-free extracts

0.2 ml. of dialyzed cell extract (see the text), 0.2 ml. of 0.1 M L-canavanine (20 μmoles), 0.2 ml. of buffer, and 0.4 ml. of water were incubated at 37° as indicated with individual experiments.

Experiment No.	pH	Buffer	Homoserine formed	Hydroxyguanidine formed
			μmoles	*μmoles*
	5.5	M/20 acetate	4.0	
1*	6.8	M/60 phosphate	9.2	
	8.2	M/60 phosphate	3.6	
2†	7.0	M/50 phosphate	4.2	4.0

* Incubation for 20 minutes.

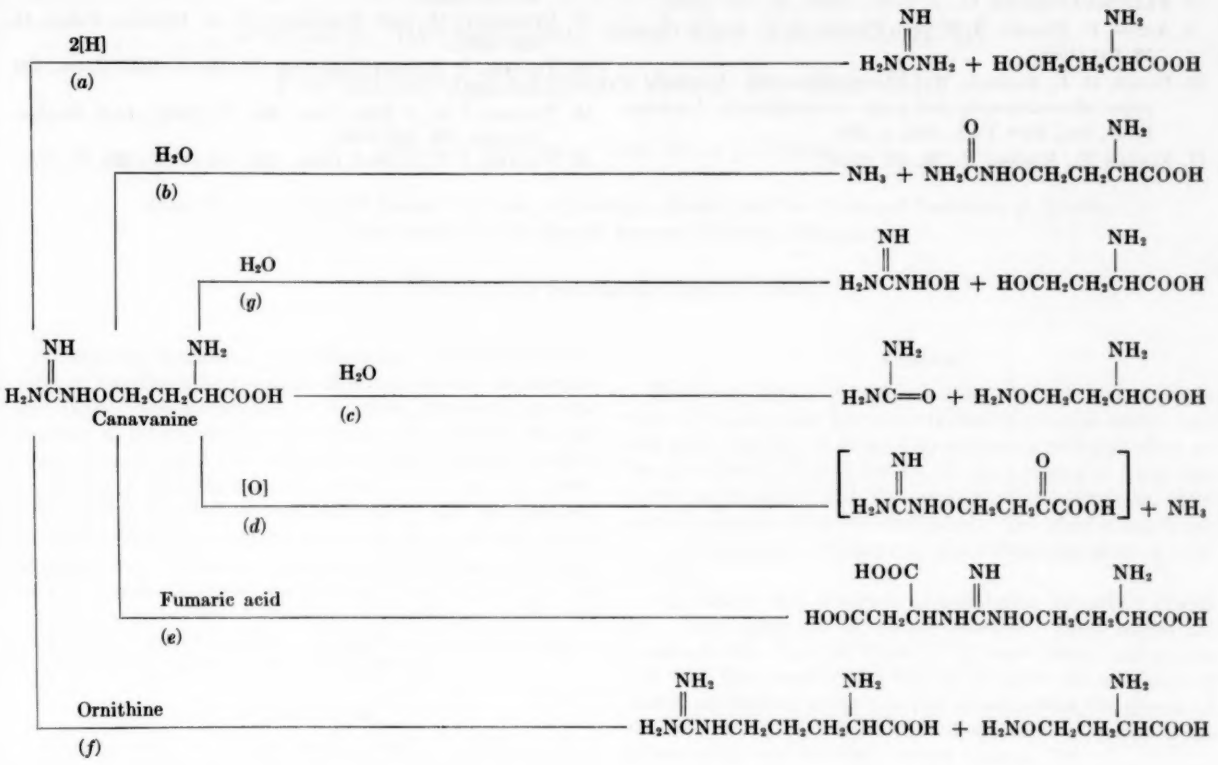
† Incubated for 1 hour. A more dilute enzyme preparation was employed in this independent experiment.

DISCUSSION

The enzymatic reactions in which canavanine is known to participate are: (*a*) reductive cleavage to homoserine and guanidine by *Streptococcus faecalis* (4); (*b*) hydrolysis to O-ureidohomoserine and ammonia by the arginine desiminase of *S. faecalis* (5); (*c*) hydrolysis to canaline and urea by arginase (13); (*d*) oxidation by L-amino acid oxidase of *Neurospora*, presumably to the corresponding keto acid and ammonia (14); (*e*) condensation with fumaric acid to yield canavaninosuccinic acid catalyzed by arginosuccinase (15); and (*f*) transamidination with ornithine (or other amidine acceptors) to yield canaline and arginine (or other guanidine compounds) by preparations from kidney (16) and *Streptomyces griseus* (7). The present investigation has revealed a third hydrolytic pathway which results in the formation of hydroxyguanidine and homoserine from canavanine (Reaction (*g*)). These known enzymatic transformations of canavanine are represented structurally in Diagram 1. Of these, Reactions (*a*) and (*g*) are the only ones that specifically require canavanine (or related derivatives of hydroxylamine) as substrates. Reactions (*b*) through (*f*) are all catalyzed either by enzymes for which arginine is a much more effective, and probably, therefore, the primary substrate, or by nonspecific enzymes such as the L-amino acid oxidase.

The possibility that reductive cleavage of canavanine by

DIAGRAM 1



S. faecalis (Reaction (*a*)) may consist in the hydrolytic cleavage (Reaction (*g*)) followed by reduction of hydroxyguanidine to guanidine has been considered. Despite the characteristic color formed when paper chromatograms of hydroxyguanidine are sprayed with the α-naphthol reagent, no formation of this product was observed in the previous work (4, 5) with growing cultures, with resting cells, or with cell-free extracts of *S. faecalis*. Attempts to show its intermediate formation in Reaction (*a*) were made during the present study, but again failed. In the presence of glucose, however, resting cell suspensions of *S. faecalis* destroy hydroxyguanidine, and, although guanidine is not a prominent product of this reaction, the possible relationship of the two pathways has not been excluded.

Hydroxyguanidine has not previously been recognized as a naturally-occurring product. The compound is formed, however, when hydroxylamine is added as a trapping agent during enzymatic transamidination (16).

The organism studied here apparently does not metabolize hydroxyguanidine further, but utilizes the homoserine formed in Reaction (*g*) for growth purposes. The metabolic reactions concerned in degradation of homoserine have not been studied in detail. Pyruvic acid was isolated (as its 2,4-dinitrophenylhydrazone) as one product of its metabolism.

SUMMARY

A pseudomonad was isolated from soil that grows readily in synthetic medium with L-canavanine as sole source of carbon and nitrogen. Growing cultures or cell-free preparations of the organism degrade canavanine by a previously undescribed route to yield hydroxyguanidine and homoserine in stoichiometric amounts. The former product accumulates in growing cultures of the organism, and the latter is utilized for growth purposes.

Procedures for isolation of hydroxyguanidine in pure form are described. This product has not been recognized previously as occurring in nature. Although not further metabolized by the organism that forms it, hydroxyguanidine is degraded to unidentified products by resting cells of *Streptococcus faecalis* in the presence of glucose.

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Dietary Methionine and the Excretion of Formiminoglutamic Acid by the Rat

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(Received for publication, July 17, 1958)

Formiminoglutamic acid is a product derived from the metabolism of histidine by mammalian liver and several microorganisms (1–3). The metabolism of FGA[1] is dependent upon the presence of tetrahydrofolic acid (4, 5), and consequently this formimino compound is excreted in the urine of human subjects or laboratory rats which have a deficiency of folic acid (6–8). Daft (6) has reported that suitable amino acid mixtures will partially replace the need for folic acid in the growth of the laboratory rat. Therefore, a study was undertaken to determine the influence of amino acids on the excretion of FGA in the urine of the rat. It was found that dietary methionine significantly reduces the excretion of FGA. We shall describe these observations and the implications derived therefrom.

EXPERIMENTAL

Weanling male rats of the Sprague-Dawley strain, weighing between 38 and 58 gm., were used. The observations recorded with diets that contained no added folic acid were made during a period of 8 to 16 weeks after the rats had been consuming this diet and the supplements indicated. The observations concerned with the influence of vitamin B_{12} were made on rats which were fed for 4 to 6 weeks after weaning the diet that lacked added vitamin B_{12}. All diets were fed *ad libitum* and rats were kept in individual cages.

The method for the determination of FGA has been described recently. However, 20 μmoles each of citric acid and $MgSO_4$ were used instead of the 200 μmoles reported (9). The latter value was published in error.

The ingredients of the "complete" diet, expressed on a per kg. basis, included the following: casein ("Vitamin Free," Nutritional Biochemicals Corporation), 90 gm.; sucrose, 670 gm.; hydrogenated vegetable oil ("MFB," Wesson Oil and Snowdrift Sales Company), 200 gm.; salts (Jones and Foster (10)), 40 gm.; and vitamins. The vitamins, in amounts added per kg. of diet, were as follows: thiamine, 30 mg.; riboflavin, 30 mg.; pyridoxine, 30 mg.; calcium pantothenate, 40 mg.; choline chloride, 800 mg.; vitamin A, 52,000 i.u.; vitamin D, 10,400 i.u.; α-tocopherol, 200 mg.; niacin, 40 mg.; biotin, 3 mg.; *i*-inositol, 800 mg.; 2-methylnaphthaquinone, 60 mg.; vitamin B_{12}, 12 μg.; and folic acid, 20 mg. For the preparation of diets lacking added folic acid or vitamin B_{12}, the vitamins concerned were omitted.

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[1] The abbreviation used is: FGA, formiminoglutamic acid.

RESULTS

The incorporation of methionine into the diet of rats which were fed a ration that had a low protein (9 per cent casein) content and contained no added folic acid has a profound effect on the excretion of FGA (Table I). In the presence of 2 per cent of the added amino acid, the excretion of the formimino compound is almost completely eliminated. The effect is significant at a concentration of 0.5 per cent and is detectable at the 0.1 per cent level.

Both the D- and L-isomers of methionine are active (Table II). They appear to be equally effective. These results are consistent with those of Wretlind (11) and Gibson and Smyth (12) who have reported (*a*) that the D-isomer of methionine is almost as effective as the L-isomer in supporting the growth of the rat (11), and (*b*) that an active racemase is present in rat kidney which converts the D- to the L-isomer (12).

A number of other amino acids[2] were examined for their influence on FGA excretion in rats maintained on the low folic acid diets. Except for homocystine and homocysteine, results with which were irregular, none of the other amino acids tested significantly reduced the excretion of FGA. Homocysteine seemed to be more effective than homocystine in reducing the excretion of the formimino compound, but in this respect its action was not as consistent as that of methionine. The results obtained with cystine and cysteine were erratic. In some trials, slight decreases in FGA excretion occurred. In others, no influence of these amino acids could be detected.

The influence of vitamin B_{12} on the excretion of FGA by the rat was examined because of (*a*) the apparent specificity of methionine; (*b*) the fact that under suitable conditions vitamin B_{12} spares the requirement for methionine in the growth of the chick, rat, and some microorganisms (13–16); and (*c*) the known requirement for vitamin B_{12} in the synthesis of methionine (17, 18). The results (Table III) indicate that deprivation of vitamin B_{12} induces FGA excretion. The amounts of formimino compound excreted after 4 weeks by rats deprived of dietary vitamin B_{12} are comparable to those excreted by animals fed diets with a low folic acid content. On the addition of vitamin B_{12} to the diet, FGA excretion was almost eliminated after 1 week (Table IV).

[2] Histidine, tryptophan, threonine, cysteine, cystine, serine, valine, phenylalanine, homocystine, homocysteine, arginine, leucine, proline, lysine, hydroxyproline, alanine, norvaline, tyrosine, glycine.

TABLE I

*Influence of added methionine on excretion of FGA by rats fed diet that lacked added folic acid**

Rat No.	Added DL-methionine		Rat No.	Added DL-methionine		Rat No.	Added DL-methionine	
	0%	2%		0%	1%		0%	0.5%
21	4.5	0.9	16	17.7	7.8	21	4.4	2.4
22	10.0	0.9	17	12.6	3.0	22	7.4	2.7
23	2.0	0.0	18	10.4	2.4	23	2.7	1.5
24	9.2	0.9	19	5.1	1.5	24	10.5	6.0
25	6.0	0.9	20	4.5	2.1	25	6.9	3.6

* Values are μmoles of FGA in urine excreted per day.

The control urine (no dietary methionine added) was obtained 1 day before the experimental collection. 24-hr. collection periods were used.

Food consumption ranged from 7 to 10 gm. per rat per day.

TABLE II

*Comparison of influence of D- and L-methionine on FGA excretion by rats fed diet that lacked added folic acid**

Rat No.	Added D-methionine		Rat No.	Added L-methionine	
	0%	0.5%		0%	0.5%
16	17.9	6.7	21	4.9	1.2
17	15.0	4.4	22	10.4	2.5
18	11.6	3.2	23	3.3	0.8
19	7.4	1.6	24	12.1	6.7
20	5.3	1.5	25	5.3	1.5

* Values are μmoles of FGA in urine excreted per day. See Table I for additional details.

TABLE III

Deprival of dietary vitamin B_{12} and FGA excretion by rats

Rat No.	"Complete" diet	Rat No.	Diet minus folic acid	Rat No.	Diet minus vitamin B_{12}
56	0.0*	51	15.9*	66	11.8*
57	2.6	52	13.2	67	14.8
58	1.0	53	3.9	68	17.0
59	0.6	54	10.6	69	17.0
60	0.0	55	7.9	70	14.8
Average food intake/rat/day..	4.6 gm.		4.8 gm.		5.0 gm.

* Values are μmoles of FGA in urine excreted per day after 4 weeks on diets.

Since FGA is derived from histidine, it could be anticipated that histidine added to the diet would give rise to an increase in FGA. Such indeed occurs (Table V). Furthermore, as in the case of the animal fed a diet that lacked added folic acid, addition of methionine to the diet with a low vitamin B_{12} content leads to a marked decrease in FGA excretion (Table V). This decrease in excretion occurred despite the increase in food intake observed with the diet that contained 1 per cent L-methionine. Methionine added to the diet effectively reduced the FGA excretion that resulted from added dietary histidine (Table V).

The addition of homocysteine to diets that contain no added vitamin B_{12} results in a diminished excretion of FGA (Table VI). The effect of homocysteine is quite marked and its magnitude approaches that of methionine. The addition of cysteine to the same diet did not consistently result in decreases.

If nicotinic acid is omitted from the "complete" diet, some growth retardation results, but FGA excretion does not occur. 15 rats were divided into three groups and fed diets that (*a*) were

TABLE IV

*Influence of dietary vitamin B_{12} on FGA excretion by rats**

Rat No.	100 μg. of vitamin B_{12} per kilo added to diet lacking vitamin B_{12}						
66	26.0	22.4	10.8	6.8	5.6	4.0	3.1
67	33.6	23.8	17.2	6.5	4.1	3.3	3.3
68	35.6	23.6	24.0	11.0	5.8	5.8	2.6
69	37.6	28.4	24.5	13.3	9.7	5.4	5.0
70	35.6	22.0	13.1	8.2	4.2	2.3	1.7
Collection day.....	1	2	3	4	5	6	7

* Values are μmoles of FGA in urine excreted per day.

Food intake was 9.9 gm. per rat per day.

Animals were fed a diet that lacked vitamin B_{12} for 7 weeks before vitamin B_{12} was added.

TABLE V

*Influence of methionine and histidine on FGA excretion by rats fed diets that lacked added vitamin B_{12}**

Rat No.	Control diet	Control diet plus 1% L-methionine	Control diet plus 0.5% L-histidine-HCl	Control diet plus 0.5% L-histidine-HCl and 1% L-methionine
81	21.6	3.1	44.4	4.8
82	21.6	1.6	79.2	5.4
83	25.0	2.6	59.0	6.0
84	16.8	2.4	87.6	7.0
85	22.9	4.6	87.6	8.3
Average food intake/rat/day....	6.8 gm.	8.7 gm.	5.9 gm.	9.9 gm.

* Values are μmoles of FGA in urine excreted per day. 3-day collection periods were used.

TABLE VI

*Effect of homocysteine and methionine on FGA excretion by rats fed diets that lacked added vitamin B_{12}**

Rat No.	Control diet	Control diet plus 1% L-homocysteine	Control diet	Control diet plus 1% L-methionine
81	21.1	13.8	21.4	8.9
82	19.4	14.0	17.8	4.0
83	27.4	15.6	22.6	5.3
84	19.4	9.7	12.7	4.7
85	23.3	14.9	21.4	8.9
Average food intake/rat/day....	6.6 gm.	6.6 gm.	5.9 gm.	7.5 gm.

* Values in μmoles FGA in urine excreted per day. 3-day collection periods were used.

TABLE VII

*Diet and weight gains**

"Complete" diet	Diet minus folic acid	Diet minus vitamin B_{12}	Diet minus folic acid and vitamin B_{12}	Diet minus niacin
135.8 (5)†	37.3 (10)	35.4 (5)	36.4 (5)	15.4 (5)
28.4 (5)	22.8 (5)	42.2 (5)		
20.0 (5)	25.0 (10)	29.4 (5)		
	39.8 (10)			
	36.6 (5)			
40.1 ‡(10)	34.6 ‡(10)			

* Gain in weight in grams during 1st month.

† Number of animals in each group enclosed in parentheses.

‡ Females.

"complete," (*b*) lacked folic acid, or (*c*) lacked niacin. The urine of each rat was examined for the presence of FGA at 4 and 6 weeks. Only those animals which were fed the diet with a low folic acid content excreted significant amounts of this compound. After the incorporation of 0.5 per cent histidine in the diet, FGA could be detected in the urine of each animal. The average values (μmoles of FGA excreted per day) found for each group at 4 weeks were the following: Group A, 0.5; Group B, 6.4; Group C, 0.5. After the incorporation of histidine into the diet, the following were found: Group A, 8.5; Group B, 43.1; and Group C, 7.0.

The weight gains made by animals fed the various regimes are shown in Table VII. With one exception (first trial), the presence or absence of folic acid or vitamin B_{12} had no influence on growth. We are unable to account for the apparent beneficial influence of these vitamins in the first trial. The omission of niacin from the diet resulted in some retardation of growth. The groups fed the "complete" diet or that lacking niacin excreted insignificant amounts of FGA; those fed the diets lacking folic acid or vitamin B_{12} did excrete significant amounts.

The FGA in the urine of the rats deprived of dietary vitamin B_{12} was characterized as a compound which (*a*) in the presence of tetrahydrofolic acid and chick liver extract and in the absence of adenosine triphosphate gave rise to N^{10}-formyltetrahydrofolic acid; (*b*) was retained by Dowex 50-H^+; (*c*) was inactivated on autoclaving at pH 6.0 to 8.0; (*d*) was degraded by alkali; (*e*) was excreted in significantly increased amounts after the ingestion of histidine (9). Fractions separated from Dowex 50-H^+ migrated on paper (in buanol-acetic acid-water) as did authentic FGA and were degraded to glutamic acid by autoclaving or alkaline treatment.

DISCUSSION

Neither folic acid nor vitamin B_{12} have any marked effect on the growth rate of the rats which were fed a 9 per cent casein diet. However, under these conditions, the two B vitamins have a demonstrable influence on the metabolism of FGA. Daft (6) has reported that rats fed a 4 per cent casein diet excrete FGA and present the typical symptoms of folic acid deficiency. More recently he has shown[3] that the conventional rat fed an 8 per cent casein diet does excrete FGA yet does not exhibit the signs of a frank folic acid deficiency. Although the rat fed an 8 to 9 per cent casein diet does not appear to have any frank deficiency symptoms directly ascribable to the absence of folic acid or vitamin B_{12}, it is nonetheless a useful animal for studies concerned with the involvement of these two vitamins in FGA metabolism.

With the data at hand, no direct conclusion can be drawn as to the mechanism through which folic acid, vitamin B_{12}, methionine, and homocysteine influence the excretion of FGA. Although the diet employed was deficient in methionine (19), retardation of growth *per se* does not appear to be a significant factor in this relationship. Neither added folic acid nor vitamin B_{12} had any great influence on growth. Furthermore, retardation of growth by the elimination of nicotinic acid from the diet did not induce excretion of FGA. It seems more likely that a specific metabolic relationship exists between FGA and methionine (or a metabolic derivative) in which the two B vitamins are involved as cofactors. A rather direct metabolic relationship is indicated by the observation that if histidine is added to a ration containing no added vitamin B_{12}, the excretion of the excess FGA is abolished almost immediately by the presence of dietary methionine (Table V).

Bennett (20) has shown that the methionine requirement for growth of the rat may be replaced by a combination of homocysteine, folic acid, and vitamin B_{12}. Recent studies by Helleiner *et al.* (21) and Kisliuk and Woods (22) directly implicate both folic acid and vitamin B_{12} in the synthesis of methionine by extracts of *Escherichia coli*. Their system involved homocysteine as the ultimate acceptor of the 1-carbon unit derived from a formaldehyde-tetrahydrofolic acid complex. In their view, vitamin B_{12} functions in the transfer of the hydroxymethyl group to homocysteine or in its final reduction to a methyl group. The present dietary studies suggest, as a possiblility, that, in the rat, a major pathway for the utilization of the available 1-carbon unit of FGA involves the over-all conversion of this unit into a methyl group via a similar reaction sequence which involves the two B vitamins and a suitable acceptor. If the normal reaction involves methionine as the acceptor, S-methylmethionine can be considered as a possible reaction product. Although the latter compound can replace methionine for growth purposes (23, 24) and is active metabolically in microbial systems (25, 26), there is no direct evidence for its natural occurrence in animal systems. If homocysteine is the normal acceptor, methionine would result. In this case the role of dietary methionine would be that of providing a source of homocysteine. Should this be so, one would have to account for the greater effectiveness of methionine as compared to its demethylation product. The reaction sequences postulated above may be blocked by the absence of either cofactor (folic acid and vitamin B_{12} derivatives) or by the absence of a suitable acceptor (methionine, homocysteine, or derived products) and FGA is excreted.

Nakao and Greenberg (27) noted that methionine in the presence of homocysteine had an activating effect on an enzyme system capable of synthesizing methionine methyl from formaldehyde or serine. The mode of action of methionine in their system was not clear. They did, however, exclude methylmethionine from consideration as an intermediate in methionine methyl synthesis from formaldehyde.

It can no longer be assumed that the occurrence of FGA in urine is a specific index of folic acid deficiency (9). Rucknagel *et al.*[4] observed the excretion of significant amounts of FGA in the

[3] Personal communication.

[4] D. L. Rucknagel, B. N. LaDu, L. Laster, J. E. Seegmiller, and F. S. Daft, personal communication.

urine of patients with pernicious anemia. The current observations with the rat confirm the findings that vitamin B_{12} deficiency induces FGA excretion.

The effect of methionine on FGA excretion in the rat may help explain the variations observed in the excretion of the formimino compound by subjects receiving anti-folic acid therapy. FGA could be detected in the urine of some treated subjects, yet appeared to be absent from the urine of others (9). It seems possible that one reason for the absence of FGA might be the presence of relatively high concentrations of methionine in the diet. Thus the extent to which FGA is excreted in the urine depends upon the degree of folic acid or vitamin B_{12} deficiency of the host, and the relative amounts of dietary histidine (the precursor of FGA) and methionine or homocysteine available. In this connection it may be noted that under the stress induced by the incorporation of extra dietary histidine, FGA excretion may be induced in the rat fed a 9 per cent casein diet that contains folic acid and vitamin B_{12}.

SUMMARY

Formiminoglutamic acid is excreted by the rat which is fed a diet that is low in protein (9 per cent casein) content and deprived of either folic acid or vitamin B_{12}. The excretion of the formimino compound is reduced by dietary methionine or homocysteine. It is postulated that a major pathway for the metabolism of the formimino compound in the intact rat involves the conversion of the available 1-carbon unit to a methyl group, a reaction sequence involving both folic acid and vitamin B_{12}. It is further postulated that an acceptor for the 1-carbon unit is supplied by methionine, homocysteine, or metabolic derivatives of these amino acids.

Acknowledgments—The authors are indebted to Drs. G. M. Briggs, F. S. Daft, R. L. Kisliuk, and J. E. Seegmiller for many helpful suggestions offered during the course of this work. We wish to thank Dr. H. G. Steinman for a generous gift of L-homocysteine.

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Effect of Vitamin B_6 Deficiency on the Basal and Adapted Levels of Rat Liver Tyrosine and Tryptophan Transaminases*

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(Received for publication, July 24, 1958)

Tyrosine-α-ketoglutarate transaminase activity in the liver was increased several-fold by the injection of hydrocortisone or L-tyrosine into the rat (1, 2). Since the activity of this enzyme was measured with an excess of its coenzyme, pyridoxal-P, it was assumed that the increase in the activity represented an increase in the concentration of the protein moiety of the transaminase. Few enzymes with dissociable coenzymes have been found to respond adaptively in their levels. The purpose of the present investigation was to determine whether the protein moiety of an enzyme could be induced under conditions which did not permit all of the enzyme molecules to acquire catalytic function, *i.e.* during deficiency of the coenzyme. For comparison, three other transaminases in the same organ were also studied. These were tryptophan-α-ketoglutarate transaminase, phenylalanine-pyruvate transaminase, and histidine-pyruvate transaminase (3).

EXPERIMENTAL

An inbred strain of Slonaker rats was used in these experiments. A group of 12 young rats with a mean body weight of 96 gm. was placed on a vitamin B_6-deficient diet obtained from the Nutritional Biochemicals Corporation. A second group of 11 rats with a mean body weight of 79 gm. was placed on a control diet which consisted of the deficient diet supplemented with 0.5 mg. of vitamin B_6 per gm. of diet. The rats were given approximately 8 gm. of food daily. After 13 to 14 weeks on the diets, half of the animals from each group were killed, and the levels of the several enzymes in their livers were assayed with and without the addition of pyridoxal-P. The remaining animals from each group were given intraperitoneal injections of 30 mg. of hydrocortisone per kg. 5 hours before they were killed. The hormone was given in 2 to 5 ml. of saline. During the 13 weeks the mean body weight of the control group increased by 120 per cent, whereas that of the deficient group increased by only 70 per cent.

The preparation and assay of the enzymes have been described in another communication (3). All the enzyme activities are expressed as micromoles of substrate transaminated per gm. of dry liver per hour at 25°. The activities of tryptophan-α-ketoglutarate transaminase have been corrected for the indolylpyruvate which disappeared during the assay. This correction amounted to approximately 15 to 30 per cent of the apparent transaminase activity (3).

* Support is acknowledged from the United States Atomic Energy Commission Grant AT(30-1)-901 to the New England Deaconess Hospital, and from the United States Public Health Service Grant A567.

RESULTS

The transaminase activities in the liver of vitamin B_6-deficient animals were lower than those of the control animals when pyridoxal-P was omitted from the assay systems (Table I, "Endogenous" data). The addition of an excess of the coenzyme to the assay system resulted in greater activation of the extracts prepared from the vitamin B_6-deficient animals than in those from the normal animals. It is notable that the degrees of suppression with the deficiency and of activation by pyridoxal-P were different in the four transaminase activities studied. The most marked effects were observed with tyrosine-α-ketoglutarate transaminase, both in the control and in the deficient extracts. Activation of phenylalanine-pyruvate transaminase by pyridoxal-P was not observed in extracts from control animals but was readily shown in extracts from deficient animals.

In the presence of an excess of the coenzyme and under the conditions of assay which obtained, the rate of transamination was proportional to the apoenzyme content. Under such conditions the mean activity of tyrosine-α-ketoglutarate transaminase was higher in extracts prepared from vitamin B_6-deficient rats, although the difference was not statistically significant ($p = 0.08$). The activities of phenylalanine-pyruvate transaminase and histidine-pyruvate transaminase in the extracts from deficient animals remained slightly but significantly lower than normal ($p < 0.05$). There was no significant difference in tryptophan-α-ketoglutarate transaminase activities in the two groups (Table I, columns 2 and 5).

Table II shows the measured activities of the apoenzymes (*i.e.* activity with excess coenzyme) of the four transaminases in the livers of the control and vitamin B_6-deficient animals 5 hours after hydrocortisone treatment. It will be seen that the administration of this hormone to the animals caused both the tyrosine-α-ketoglutarate apotransaminase and tryptophan-α-ketoglutarate apotransaminase activities in the liver to increase. Furthermore, the adaptive increases of these two apoenzymes were not impaired by the deficiency of the coenzyme. Hydrocortisone was without significant effect on the levels of phenylalanine-pyruvate apotransaminase and histidine-pyruvate apotransaminase in the liver.

TABLE I

Basal levels of four liver transaminases in control and vitamin B_6-deficient rats

Transaminases	5 control animals			6 deficient animals		
	Endogenous* Mean ± s.e.	With coenzyme† Mean ± s.e.	Activation	Endogenous* Mean ± s.e	With coenzyme† Mean ± s.e.	Activation
			%			%
Tyrosine-α-ketoglutarate	18 ± 3.9	73 ± 17	310	10 ± 1.9	118 ± 13	1080
Tryptophan-α-ketoglutarate	13 ± 1.3	16 ± 2.2	23	9 ± 0.8	15 ± 1.4	67
Phenylalanine-pyruvate	100 ± 9.4	107 ± 7.6	7	28 ± 5.3	67 ± 7.3	140
Histidine-pyruvate	33 ± 5.0	43 ± 4.0	30	15 ± 2.0	27 ± 3.7	80

* No pyridoxal-P added to the assay mixture.
† With excess pyridoxal-P in assay.

TABLE II

Effect of hydrocortisone on levels of four transaminases in livers of control and vitamin B_6-deficient rats

Both control and deficient animals were given injections of hydrocrotisone. The levels of the transaminase in the livers of animals 5 hours after the treatment are given below. All the activities designated in this table were measured in the presence of an excess of pyridoxal-P (determination of the apoenzyme content). The activities of the hormone-treated control animals were compared (per cent change) with those of the untreated controls (Table I, column 2), and the activities of the hormone-treated deficient animals were compared with those of the untreated deficient animals (Table I, column 5). The statistical significance of the differences was evaluated by the *t* test.

Transaminases	Control animals		6 deficient animals	
	Mean ± s.e.	Per cent change	Mean ± s.e.	Per cent change
Tyrosine-α-ketoglutarate	370 ± 53	+400, $p < 0.001$	500 ± 86	+325, $p < 0.01$
Tryptophan-α-ketoglutarate	37 ± 2.9	+132, $p < 0.001$	34 ± 4.4	+126, $p < 0.01$
Phenylalanine-pyruvate	120 ± 9	+12, not significant	80 ± 15	+19, not significant
Histidine-pyruvate	49 ± 4.1	+14, not significant	31 ± 2.9	+15, not significant

DISCUSSION

Studies of the concentrations of an apoenzyme maintained by cells during deficiency of the coenzyme might reveal the presence of an important homeostatic mechanism. Such a mechanism would increase the apoenzyme concentration whenever the coenzyme concentration decreases so that the concentration of the active enzyme would be maintained. Only two pyridoxal-P-requiring enzyme systems which have been studied showed increases in the apoenzyme concentrations under conditions of vitamin B_6 deficiency. The cellular concentrations of diaminopimelic apodecarboxylase and lysine apodecarboxylase in a vitamin B_6-requiring *Escherichia coli* mutant grown in trace amounts of pyridoxine were found to be twice as high as those in cells grown in a pyridoxine-rich medium (4). Tyrosine apodecarboxylase was produced by *Streptococcus faecalis* R in cells containing little or none of the coenzyme, but the relationship of the apoenzyme content to the quantity of pyridoxal-P available was not studied (5). The levels of apokynureninase (6) and 3,4-dihydroxyphenylalanine apodecarboxylase (7) in rat liver, of glutamic apodecarboxylase in rat brain (8), and of glutamic-oxalacetate apotransaminase and glutamic-pyruvate apotransaminase in rat liver (9) and duck ventricle (10) were not significantly affected by vitamin B_6 deficiency. The levels of cysteine sulfinic acid apodecarboxylase in rat liver (11, 12), of glutamic-oxalacetate apotransaminase in rat kidney and heart (13), and of 5-hydroxytryptophan apodecarboxylase in rat kidney (14) decreased in vitamin B_6 deficiency. The results from the present studies indicated that there was a possibly significant compensatory increase in the apoenzyme concentration of tyrosine-α-ketoglutarate transaminase during deficiency of the coenzyme. Under the same conditions no increases were observed in the concentrations of three other apotransaminases: tryptophan-α-ketoglutarate, phenylalanine-pyruvate, and histidine-pyruvate. It may be significant that the levels of the two apotransaminases which could not be adaptively increased fell during the deficiency of the coenzyme, whereas the levels of the two apotransaminases which could be adaptively increased remained the same or rose with the deficiency.

During the course of the present investigation the protein moiety of a new enzyme, tryptophan-α-ketoglutarate transaminase, was found also to be increased in the liver by the administration of hydrocortisone. The increases of this apotransaminase and of the tyrosine-α-ketoglutarate apotransaminase induced by hydrocortisone were not affected by vitamin B_6 deficiency in the animal. This suggested that the protein moieties of these enzymes were produced by the liver cells at unimpaired rates even when the molecules could not be completely functional because of the lack of the coenzyme. It was recently reported that the protein moiety of another liver enzyme, glutamic-pyruvate apotransaminase, could also be increased by the administration of hydrocortisone and that the increase was not affected by vitamin B_6 deficiency in the rat (9).

The different adaptive behavior of the apotransaminases and the different degrees of activation *in vitro* by pyridoxal-P confirmed other evidence, not presented, that four separate enzymes

were involved in the transaminations studied. The 4- to 5-fold increase in the tyrosine-transaminating activity caused by the administration of hydrocortisone distinguished this enzyme from the transaminase acting on tryptophan, which increased only 2- to 3-fold in the same livers. In all preparations the addition of pyridoxal-P produced greater activation of the tyrosine transaminase than of the tryptophan transaminase. The phenylalanine-pyruvate and histidine-pyruvate transaminase were set apart from the former two enzymes by their lack of response to hydrocortisone. The failure of pyridoxal-P to activate the phenylalanine-pyruvate transaminase activity unless the extracts were prepared from vitamin B_6 deficient rats indicated that this enzyme was different from the one that acted on histidine. The latter was activated by pyridoxal-P even in extracts prepared from normal rats (Table I).

SUMMARY

1. The activities of four different transaminases, tyrosine-α-ketoglutarate, tryptophan-α-ketoglutarate, phenylalanine-pyruvate, and histidine-pyruvate were studied in the livers of control and vitamin B_6-deficient rats. The lowered activities of these enzymes under conditions of vitamin B_6 deficiency were primarily attributable to a depletion of the coenzyme, pyridoxal phosphate, and not to significant alteration of the concentrations of the protein moieties of these enzymes.

2. The concentration of tryptophan-α-ketoglutarate apotransaminase in the liver, like that of the tyrosine-α-ketoglutarate apotransaminase, was increased by hydrocortisone treatment of the animals. The concentrations of phenylalanine-pyruvate and histidine-pyruvate apotransaminases were not changed by hydrocortisone treatment.

3. The increases of the protein moeities of tyrosine-α-ketoglutarate and tryptophan-α-ketoglutarate transaminases induced by hydrocortisone were not affected by deficiency of the coenzyme in the animal.

Acknowledgment—We should like to thank Professor A. B. Hastings for reading and commenting on this manuscript.

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Specificity of the Adaptive Response of Tyrosine-α-ketoglutarate Transaminase in the Rat*

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(Received for publication, July 24, 1958)

The tyrosine-α-ketoglutarate transaminase of liver was increased several-fold 5 hours after a single injection of hydrocortisone was given to either intact or adrenalectomized rats. In contrast, the injection of a substrate of this enzyme, L-tyrosine, increased the enzyme level in only the intact animals as opposed to the adrenalectomized ones, unless hydrocortisone was also administered. Tyrosine administered together with hydrocortisone produced an increase of the enzyme in the adrenalectomized animals which was twice as high as that obtained by the administration of hydrocortisone alone (1). It was concluded that the presence or release of adrenal corticoid was a necessary condition for the enzyme level to respond adaptively to the substrate.

We wish to report here further experiments designed to test (*a*) whether the adaptive behavior of this enzyme is tissue-specific; (*b*) whether the level of this enzyme is specifically affected by its substrate, L-tyrosine; (*c*) whether the levels of *p*-hydroxyphenylpyruvate oxidase and homogentisate oxidase, the next two enzymes involved in the oxidative degradation of tyrosine, are under the same type of control that affects the tyrosine-α-ketoglutarate transaminase level; and finally (*d*) whether the level of another transaminase, phenylalanine-pyruvate transaminase, is also controlled by hydrocortisone and by one of its substrates.

EXPERIMENTAL

Animals—Albino rats from the inbred strain of Slonaker rats reared in our laboratory at the Cancer Research Institute were used in all the experiments. The animals were provided with Purina chow pellets *ad libitum*. The materials for injection included saline suspensions of L-tyrosine, corticosterone, and cortisone; hydrocortisone was administered as the soluble sodium hemisuccinate (Solu-Cortef). The dose was 2 to 5 ml. of neutralized solutions which were approximately isotonic, and the route of injection in all cases was intraperitoneal.

Reagents and Enzyme Assays—All chemicals were obtained from commercial sources. The keto-enol tautomerase was prepared in this laboratory (2). The procedure for the preparation of liver extracts has been described in an earlier paper (3). Extracts of all other tissues were made by grinding the chopped tissues in a ground glass homogenizer. The intestine was first cut open and thoroughly rinsed with tap water. The remaining steps of preparation were the same as those applied to the liver. The procedure for the determination of tissue dry weights has been previously described (1). Tyrosine-α-ketoglutarate transaminase, phenylalanine-pyruvate transaminase, and *p*-hydroxyphenylpyruvate oxidase were assayed according to the procedures described in a previous paper (3). The possibility that the increase in tyrosine-α-ketoglutarate transaminase represents the formation of a second enzyme normally not present in the liver was investigated by comparison of the Michaelis constants for L-tyrosine and α-ketoglutarate and the pH optimum of the transaminase prepared from a control animal with similar data on an animal which had received an injection of L-tyrosine 5 hours before the experiment. No significant differences in these properties of the transaminases from the two animals were observed, although the activity per gm. of tissue was 8 times higher in the animals that received injections of tyrosine. Homogentisate oxidase was assayed by a manometric method (4). The rate of oxygen uptake was proportional to the amount of homogentisate oxidase over a 4-fold range.

RESULTS

Tissue Distribution of Enzymes—The basal levels of the four enzymes in the liver, kidney, and heart are given in Table I. It is clear that the activities of these enzymes in the liver greatly exceeded the activities of those in the other two organs, both on the "per organ" and on the "per gm. of tissue" bases. Several other tissues, including the brain, intestine, lung, muscle, and spleen were also examined but were found to contain no appreciable amounts of these enzymes.

The great abundance of these enzymes in only the liver suggested that their total activities in this organ would determine the capacity of the whole organism to carry out the associated metabolic reactions.

Tissue Specificity in Response of Tyrosine-α-ketoglutarate Transaminase—To determine whether the ability of tyrosine-α-ketoglutarate transaminase to respond adaptively was tissue-specific, the level of this enzyme was measured in both the liver and the kidney after administration of two kinds of stimuli. First, two intact rats were given injections of L-tyrosine. After 5 hours the levels of this enzyme in the livers of these two animals were found to be increased 6-fold over the control values. In contrast, the activities of this enzyme in the kidneys remained unchanged. Similarly the level of this enzyme in the kidneys of an adrenalectomized rat which received both L-

* Support is acknowledged from United States Atomic Energy Commission Grant AT(30-1)-901 to the New England Deaconess Hospital, from United States Public Health Service Grant A567, and from the Milton Fund.

TABLE I

Tissue distribution of four enzymes in rats

Tissue	Phenylalanine-pyruvate transaminase		Tyrosine-α-ketoglutarate transaminase		p-Hydroxy-phenylpyruvate oxidase		Homogentisate oxidase	
	Per gram of tissue*	Total organ†	Per gram of tissue	Total organ†	Per gram of tissue	Total organ	Per gram of tissue	Total organ
Liver...	39	100	36	100	74	100	217	100
Kidney.	4	4	1	2	3	1	70	13
Heart..	6	2	4	2	5	1	7	1

* The activities are expressed as micromoles of substrate converted per gm. of fresh tissue per hour, at 25° for the first three enzymes and at 38° for homogentisate oxidase.

† The total organ activities were calculated by multiplying the activity per gm. by the organ weight and are expressed as relative units with the liver assigned an activity of 100.

tyrosine and hydrocortisone by injection remained unchanged, despite the fact that the level in the liver had increased 10-fold.

Specificity of L-Amino Acids as Stimuli—Table II shows the levels of tyrosine-α-ketoglutarate transaminase in the liver 5 hours after the injection of L-tyrosine, L-glutamate, or L-phenylalanine (second column). It can be seen that administration of L-glutamate and L-phenylalanine did increase the level of this transaminase, although the increases were considerably smaller than that produced by L-tyrosine. We also observed a tyrosine-α-ketoglutarate transaminase level of 390 in a rat which was similarly given an injection of L-valine and a level of 810 in an L-alanine-treated one. It is noteworthy that although L-glutamate is also a substrate of tyrosine-α-ketoglutarate transaminase, its effect on the level of this enzyme was not significantly greater than the effects of the nonsubstrate amino acids tested. Since the administration of hydrocortisone to intact animals also increased the level of this enzyme several-fold (see next section), the effects produced by these other amino acids might be attributed simply to the condition of stress created by the injection of large quantities of a single amino acid. The term stress is used to denote the physiological state of augmented internal secretion of adrenal cortical hormones. That a nonspecific stress-producing agent could actually increase the level of tyrosine-α-ketoglutarate transaminase was further supported by the results obtained with a compound which is unrelated to tyrosine metabolism. Injections of propylene glycol in doses of 0.5 ml. per 100 gm. of body weight caused the level of this enzyme to increase to an average of 1390 units in three intact rats. All six adrenalectomized rats given this dose of propylene glycol died within 5 hours.

Table II also lists the activities of the other three enzymes found after the amino acids were given intraperitoneally. Phenylalanine-pyruvate transaminase and p-hydroxyphenylpyruvate oxidase levels were not significantly changed by the amino acids. Homogentisate oxidase level was elevated, although the increments were considerably smaller than the changes of tyrosine-α-ketoglutarate transaminase.

Time Courses of Response to Hydrocortisone and L-Tyrosine—Both in the studies previously reported (1) and in the experiments summarized in Table II of the present report, the animals were killed 5 hours after a compound was administered. This time was chosen because the responses of tyrosine-α-ketoglutarate transaminase to the given doses of both hydrocortisone and L-tyrosine were found to be maximal at this point. The temporal course of response to hydrocortisone over a period of 12 hours can be seen in Fig. 1. For comparison the levels of the three other enzymes in the liver are also given. Both the tyrosine-α-ketoglutarate transaminase and the homogentisate oxidase levels were elevated by the treatment with hydrocortisone as was the result in the case of injections of amino acids. Both enzymes reached a peak level in approximately 5 hours. The levels of p-hydroxyphenylpyruvate oxidase and phenylalanine-pyruvate transaminase remained fairly constant during the entire period.

Time studies on the tyrosine-α-ketoglutarate transaminase levels after the injection of L-tyrosine were complicated by two factors. First, the poorly soluble amino acid must be administered in a suspension. Since the absorption of the compound was expected to be more gradual the effect should be of longer duration. Second, the administration of the amino acid was expected to cause some release of adrenal cortical hormones which would be added to the effect of the substrate itself (1). Fig. 2 shows the levels of tyrosine-α-ketoglutarate transaminase after a single injection of L-tyrosine. It can be noted that the maximal increase with the substrate was much greater than that produced by the administration of hydrocortisone. In the case of L-tyrosine-treated animals, the level of the enzyme also remained high for a longer period of time.

Effects of Corticosterone and Cortisone—Finally it should be mentioned that in addition to hydrocortisone, corticosterone and cortisone were also effective in increasing the level of tyrosine-α-ketoglutarate transaminase in the liver. Two rats which received injections of corticosterone (30 mg. per kg.) had tyrosine transaminase levels which were 490 and 300 per cent higher

TABLE II

Response of enzyme levels in liver to L-amino acids

The activities are expressed as micromoles of substrate converted per gm. of dry liver per hour, and each figure represents the mean of the group measured. The standard error is also given, and the figure in brackets represents the number of animals. All assays were carried out at 25°, except that of homogentisate oxidase which was measured at 38°. All amino acids were administered according to a dosage of 3.3 mmoles per kg., and the treated animals were killed 5 hours afterward.

Treatment	Phenylalanine-pyruvate transaminse	Tyrosine-α-ketoglutarate transaminase	p-Hydroxy phenylpyruvate oxidase	Homogentisate oxidase
Control.....	129 ± 7 [12]	159 ± 21 [23]	260 ± 25 [16]	565 ± 22 [15]
L-Tyrosine.....	178 ± 60 [3]	1370 ± 168 [8]	236 ± 17 [5]	840 ± 49 [3]
L-Glutamate.....	139 ± 12 [4]	600 ± 187 [4]	256 ± 30 [4]	764 ± 74 [2]
L-Phenylalanine.....	200 [1]	304 ± 72 [3]		

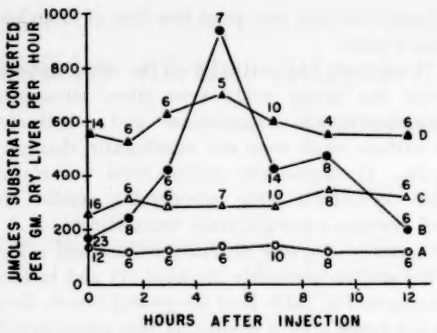

FIG. 1. Time of response of phenylalanine-pyruvate transaminase (○——○), tyrosine-α-ketoglutarate transaminase (●——●), *p*-hydroxyphenylpyruvate oxidase (△——△), and homogentisate oxidase (▲——▲), in the rat liver. All animals received injections of hydrocortisone according to a dosage of 30 mg. per kg. The mean value of each group is plotted, and the number of animals in the group is indicated by the numeral at each point. All enzymes were assayed at 25° except homogentisate oxidase which was measured at 38°.

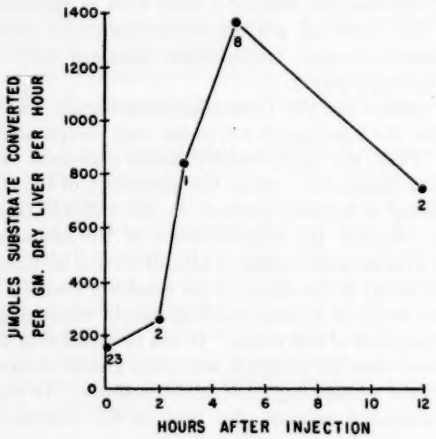

FIG. 2. Time of response of tyrosine-α-ketoglutarate transaminase in the rat liver to L-tyrosine administered in a dose of 3.3 mmoles per kg. The mean value of each group is plotted, and the number of animals in the group is indicated by the numeral at each point.

than the basal value, whereas the level of this enzyme in two other rats treated with cortisone (30 mg. per kg.) were 650 and 440 per cent higher than the basal level. Corticosterone failed to increase the levels of phenylalanine-pyruvate transaminase and *p*-hydroxyphenylpyruvate oxidase.

DISCUSSION

The distribution of tyrosine-α-ketoglutarate transaminase found in different tissues of the rat was essentially like the distribution of this enzyme found in the tissues of the dog (5). The distribution of homogentisate oxidase in different tissues of the rat reported here supported the findings of a previous study of the same species (6). In all these studies liver was found to be the only organ rich in the two enzymes.

Although all of the four enzymes studied showed very similar tissue distribution in that all were almost exclusively located in the liver, their responses to individual stimuli were quite different in the same organ. Thus the levels of tyrosine-α-ketoglutarate transaminase and homogentisate oxidase underwent adaptive changes in contrast to the stability of the levels of phenylalanine-pyruvate transaminase and *p*-hydroxyphenylpyruvate oxidase.

Different adaptive behaviors of two other transaminases in the rat liver were recently reported. Glutamic-pyruvate transaminase was increased several-fold by hydrocortisone administration whereas the level of glutamic-oxaloacetate transaminase remained unchanged (7). The physiological significance of the different adaptive behaviors of the transaminases remains to be explored.

Comparison of the effects of injections of several L-amino acids indicated that L-tyrosine exerted the greatest effect on the level of tyrosine-α-ketoglutarate transaminase. The increases of the level of this enzyme produced by the other amino acids were considerably smaller than those produced by L-tyrosine and could probably be attributed to the release of adrenal cortical hormones caused by the introduction of large quantities of a single amino acid. The greater increase produced by L-tyrosine would be the result of two superimposed effects: the release of adrenal cortical hormones and the specific effect of substrate induction. That the two effects could be summated was shown earlier by the administration of both L-tyrosine and hydrocortisone to adrenalectomized rats (1).

The parallel increases of homogentisate oxidase and tyrosine-α-ketoglutarate transaminase, after the injection of hydrocortisone and of amino acids, superficially resembled the phenomenon of simultaneous enzyme induction frequently observed in microbial systems (8). However, under certain experimental conditions the level of homogentisate oxidase remained low even when large quantities of homogentisate were excreted by the animals and when the tyrosine-α-ketoglutarate transaminase levels in these animals were high (4). This strongly suggested that the level of homogentisate oxidase in the liver is more reliable as an index of the amount of circulating adrenal cortical hormones than as an index of the abundance of homogentisate in the animal. Parallel changes in *p*-hydroxyphenylpyruvate oxidase levels have not been detected with changes of tyrosine-α-ketoglutarate transaminase. The presence of normal levels of maleylacetoacetate isomerase and fumarylacetoacetate hydrolase in the liver in a human subject with alkaptonuria devoid of homogentisate oxidase indicated that the fourth and fifth enzymes in the pathway of tyrosine degradation were also under independent control (9). The absence of the phenomenon of simultaneous adaptation was also noted in the tryptophan oxidation system in the liver. Neither formylase (10) nor kynureninase (11) was increased by the administration of L-tryptophan.

It is noteworthy that although hydrocortisone and cortisone are well known for their general protein-catabolic effects, there are enzymes whose levels were actually increased after adrenal cortical hormone treatment (12). Examples are D-amino acid oxidase (13), arginase (14), glutamic-pyruvate transaminase (7, 15), tryptophan peroxidase (16, 17), and xanthine oxidase (18). To this list may be added tyrosine-α-ketoglutarate transaminase and homogentisate oxidase. It is important to note that with one exception all of these enzymes are involved in the catabolism of amino acids. Whether the increases of these

enzymes are causes or effects of general protein breakdown remains a very important question.

Finally, the 6- to 10-fold increase in tyrosine-α-ketoglutarate transaminase levels in 5 hours and the rapid restoration to a normal level in approximately 12 hours indicated that the potential turnover rate of this lever protein was much more rapid than the average rate of liver proteins which were estimated to have a half-time of 7 days in the rat (19). The half-time of tyrosine-α-ketoglutarate transaminase was probably less than 3 hours under the experimental conditions. Such rapid changes in the level of a specific protein have also been observed in the case of tryptophan peroxidase (20).

SUMMARY

1. Liver was found to be the only organ rich in tyrosine-α-ketoglutarate transaminase, phenylalanine-pyruvate transaminase, *p*-hydroxyphenylpyruvate oxidase, and homogentisate oxidase in the rat.

2. The tyrosine-α-ketoglutarate transaminase in the liver, but not in the kidney, increased adaptively with administration of L-tyrosine and hydrocortisone. The level of this enzyme in the liver was also increased by the administration of corticosterone and cortisone.

3. Tyrosine-α-ketoglutarate transaminase in the liver was increased most effectively by the injection of its substrate, L-tyrosine. The other L-amino acids given by injection produced much smaller increases, which were attributable to adrenal cortical stress.

4. The level of homogentisate oxidase in the liver could also be increased adaptively and seemed to be predominantly under the control of adrenal cortical hormones.

5. Phenylalanine-pyruvate transaminase and *p*-hydroxyphenylpyruvate oxidase levels were not increased by the administration of hydrocortisone, corticosterone, or amino acids.

6. The maximal increases in both tyrosine-α-ketoglutarate transaminase and homogentisate oxidase were observed 5 hours after the injection of hydrocortisone.

Acknowledgments—The authors wish to thank Professor A. B. Hastings for helpful comments on the preparation of this manuscript and Dr. J. O. Lawrence of The Upjohn Company for sending us a generous supply of corticosterone.

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Effect of Glucose and Various Nucleosides on Purine Synthesis by Ehrlich Ascites Tumor Cells *in Vitro**

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(Received for publication, July 7, 1958)

The pattern of formate-C^{14} incorporation by Ehrlich ascites tumor cells has been found to be remarkably different *in vitro* from the pattern of incorporation *in vivo* (3–5). Thus, after incubation of cells with formate-C^{14} *in vitro*, the specific activity of the DNA thymine was 10 to 30 times higher than that of the DNA purines. In contrast, after incorporation of formate-C^{14} by these cells *in vivo*, the specific activity of DNA thymine was similar to that of the DNA purines. The deficiency in purine synthesis *de novo* in the system *in vitro* could be partially alleviated by the addition of AIC[1] (3) or pigeon liver extract (4). However, addition of these substances did not influence the incorporation of P^{32} into nucleic acids or of formate-C^{14} into DNA thymine (3). The present report is concerned primarily with the effect of glucose and various other compounds on formate-C^{14} incorporation into purines in the system *in vitro*.

EXPERIMENTAL

The Ehrlich ascites tumor was maintained by weekly transfer of 2×10^7 tumor cells into the intraperitoneal cavities of mice of CF_1 strain or from laboratory stock. 7 days after inoculation the mice were killed, and the tumor cells were harvested and washed several times in cold Krebs-Ringer-phosphate or bicarbonate. The cells were suspended in the buffer, and aliquots containing 2 to 3×10^8 cells in 3 ml. were incubated for 3 hours at 37° in the presence of formate-C^{14} (2 to 4 μmoles per approximately 10^6 c.p.m. per flask) and various other additions as indicated below. Phenol red was included in the medium, and the pH was kept at 7.4 by addition of isotonic HCl or NaOH. After incubation the cells were extracted with cold $HClO_4$ and ethanol-ethyl ether. Nucleic acids were extracted with 10 per cent NaCl and precipitated from the salt extract with ethanol. After incubation for 18 hours at 37° in the presence of 0.3 N KOH, DNA was separated from RNA by acid precipitation (6). The purine- and pyrimidine-containing compounds of the acid-soluble fraction were adsorbed on activated Norit and eluted with 50 per cent ethanol-NH_4OH (in a ratio of 100:1). Preliminary assays of ultraviolet absorption and radioactivity were then carried out on the acid-soluble charcoal eluate and the RNA and DNA fractions. These fractions were then evaporated to dryness over H_2SO_4 and were hydrolyzed with 0.1 ml. of 70 per cent $HClO_4$ for 1 hour at 100°. The free purines and pyrimidines were separated by two-dimensional chromatography in (*a*) isopropanol-HCl-H_2O and (*b*) *n*-butanol-NH_4OH-H_2O according to the method of Wyatt (7). The ultraviolet-absorbing spots were cut out and sewed to strips of paper and a descending chromatograph was run in distilled water (8). The compounds were then located under ultraviolet light and eluted. The eluates were assayed for ultraviolet absorption and radioactivity. Radioactivity was determined by plating infinitely thin samples on stainless steel planchets and counting with an end window Geiger counter or a gas flow counter fitted with an ultrathin window. AIC was determined by a modification of the Bratton-Marshall-test (9).

RESULTS AND DISCUSSION

Earlier work in this laboratory (3) has shown that AIC markedly stimulated the incorporation of formate-C^{14} into the purines of the ascites tumor *in vitro*. Various other compounds were then tested for a similar effect. As shown in Table I, the addition of glucose was found to stimulate the incorporation of formate-C^{14} into RNA purines to a level 13 times that of the control. Glutamine also had a slight stimulatory effect, but succinate, aspartate, DPN, phosphoenol pyruvate, and phosphoglyceric acid were not significantly effective. The effect of glucose on the incorporation of formate-C^{14} into acid-soluble adenine and into RNA at various intervals during the incubation period is shown in Fig. 1. The stimulation was evident within the first half-hour and was maintained throughout the 3-hour incubation period. The effect of glucose on the incorporation of formate-C^{14}, adenine-8-C^{14}, and glycine-2-C^{14} into nucleic acid purines and thymine is shown in Table II. The incorporation of all three precursors into the nucleic acid purines was markedly stimulated, but the incorporation of formate-C^{14} into DNA thymine was not significantly affected.

This stimulatory effect of glucose on purine formation could be the result of several factors. Thus, glucose could provide a source of energy, a source of purine ring precursors, as does AIC, or a source of ribose phosphate or PP-ribose-P. Since succinate, aspartate, phosphoenol pyruvate, and phosphoglycerate did not influence the incorporation of formate-C^{14}, and since glucose itself stimulated neither the incorporation of formate-C^{14} into DNA thymine, nor the incorporation of P^{32} into nucleic acids (3) nor oxygen uptake (3), it seems unlikely that

* The initial work described in this paper was carried out while the author was privileged to work in the Department of Biochemistry, University of Glasgow, Scotland. Preliminary reports have been presented previously (1, 2).

[1] The abbreviations used are: AIC, 4 (or 5)-amino-5 (or 4) imidazole carboxamide; PP-ribose-P, 5-phospho-α-ribosyl-pyrophosphate.

TABLE I

Effect of various compounds on incorporation of formate into purines by ascites tumor cells

2.26×10^8 to 3.26×10^8 washed cells were suspended in 3 to 4 ml. of Krebs-Ringer-bicarbonate buffer and incubated with formate-C^{14} (2 to 4 μmoles per 3.6 to 4.5×10^6 c.p.m. per flask).

Addition	Specific activity, % control	
	ASCE*	RNA
None	100	100
Glucose (20 μmoles)	900	1270
Succinate (20 μmoles)	97	174
Aspartate (20 μmoles)	126	111
DPN (4 μmoles)	43	71
Phosphoenolpyruvate (20 μmoles)	72	89
Phosphoglycerate (20 μmoles)	80	102
Glutamine (20 μmoles)	157	282
Glutamine (50 μmoles)	204	297

* Acid-soluble charcoal eluate.

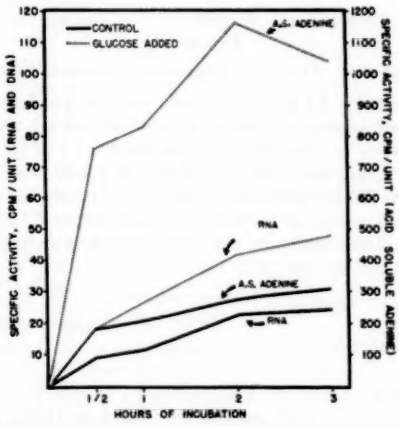

FIG. 1. Incorporation of formate-C^{14} at various intervals ± glucose. 2.3×10^8 washed cells were suspended in 3 ml. of Krebs-Ringer bicarbonate buffer and incubated for various intervals in the presence of formate-C^{14} (4 μmoles, 1.76×10^6 c.p.m. per flask) and ± 20 μmoles of glucose. *A.S. Adenine* = acid-soluble adenine.

the stimulation was entirely the result of an effect on energy production.

Stewart and Sevag (7) have reported that AIC accumulated during the metabolism of glucose by *Escherichia coli*. However, no such accumulation was found in the ascites tumor cells with or without glucose (13 μmoles) and/or *A*-methopterin (40 to 200 μg.). This lack of accumulation, in addition to the stimulatory effect of glucose on the incorporation of the preformed purine, adenine-8-C^{14}, indicated that the stimulation produced by glucose was not primarily a result of the formation of purine ring precursors.

It seemed possible that the stimulatory effect of glucose was a result of its use as a source of ribose phosphate or PP-ribose-P necessary for the formation of purine nucleotides (10–12). Various ribose-containing compounds were therefore tested at different concentrations. As shown in Fig. 2, AIC-riboside was much more effective than free amino-imidazolecarboxamide. Ribose and ribose-5-phosphate were ineffective even when as much as 100 μmoles were added per flask. However, it is possible that the cells lack ribokinase and are impermeable to phosphorylated sugars.

The magnitude of the stimulation produced by AIC-riboside in comparison to AIC suggested that the addition of ribose (in combined, activated, or available form) is of great importance in purine synthesis *de novo* by these cells *in vitro*. As shown in Table III, the combination of glucose and AIC was more stimulating than AIC alone, whereas the combination of glucose and AIC-riboside was not significantly more effective than the latter alone. These results suggest that both the

TABLE II

Effect of glucose on incorporation of formate-C^{14}, glycine-2-C^{14}, and adenine-8-C^{14} into nucleic acid bases

Approximately 8×10^8 unwashed cells suspended in ascitic fluid were incubated for 3 hours in the presence of formate-C^{14} (4 μmoles per 5×10^6 c.p.m. per flask), adenine-8-C^{14} (52 μmoles per 2.6×10^6 c.p.m. per flask), or glycine-2-C^{14} (40 μmoles per 5×10^6 c.p.m. per flask).

Precursor	Medium	Specific activity in c.p.m./μmole				
		RNA		DNA		
		Adenine	Guanine	Adenine	Guanine	Thymine
Formate-C^{14}	Ascitic fluid	457	904	53.7	409	4895
	Ascitic fluid + glucose (27 μmoles)	3480	8120	673	2185	5540
Adenine 8-C^{14}*	Ascitic fluid	1320	45	50	27	
	Ascitic fluid + glucose (22 μmoles)	6880	1063	1220	216	
Glycine-2-C^{14}*	Ascitic fluid	1.5	190	44	13	
	Ascitic fluid + glucose (22 μmoles)	990	1880	72	354	

* These data were collected in collaboration with Dr. R. Y. Thomson, Department of Biochemistry, University of Glasgow.

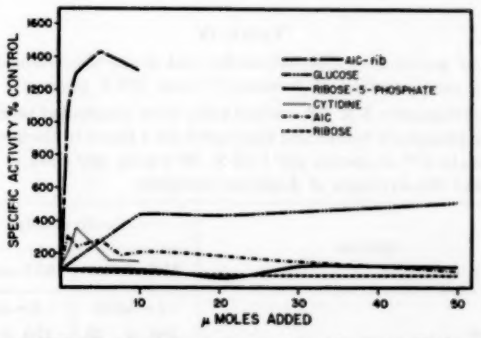

FIG. 2. Effect of various compounds on formate-C^{14} incorporation into RNA by ascites tumor cells *in vitro*. Approximately 2.5×10^8 washed cells were suspended in 3 ml. of Krebs-Ringer phosphate buffer and incubated 3 hours in the presence of formate-C^{14} (2 μmoles, approximately 10^6 c.p.m. per flask), with additions as indicated. *AIC-rib* = AIC riboside.

latter compound and glucose provide a source of activated or available ribose, which stimulates the synthesis of purines *de novo* by ascites tumor cells *in vitro*.

The pyrimidine ribonucleosides, cytidine and uridine, also stimulated the incorporation of formate-C^{14} into purines, but the deoxyribonucleosides of these pyrimidines were ineffective (Table IV). Cytidine stimulated the incorporation of formate-C^{14} not only into RNA purines but also into the purines of the acid-soluble and DNA fractions. Deoxycytidine, however, did not stimulate the incorporation of formate-C^{14} into the purines of any fraction (Table V). Thus, these cells seem able to utilize pyrimidine ribonucleosides but not deoxyribonucleosides as a source of pentose for the formation of both purine ribonucleosides and deoxyribonucleosides. The transfer of the ribose to purine intermediates may occur by means of nucleoside phosphorylase reactions or transglycosidation (13-15). The formation of purine deoxyribonucleosides could occur either by direct reduction of the ribonucleoside, or by transfer of the labeled purine to endogenous deoxyribose (14). Both cytidine and deoxycytidine stimulated the incorporation of formate-C^{14} into thymine (Table V), as has also been reported previously by

TABLE III

Effect of glucose in combination with AIC or AIC-riboside on incorporation of formate-C^{14} into purines

In Experiment I 2.3 × 10^8 washed cells were suspended in 4 ml. of Krebs-Ringer-bicarbonate buffer and incubated for 3 hours at 37° in the presence of formate-C^{14} (4 μmoles per 2.6 × 10^6 c.p.m. per flask). In Experiment II, 2.8 × 10^8 washed cells were suspended in 3 ml. of Krebs-Ringer-bicarbonate buffer and incubated for 3 hours at 37° in the presence of formate-C^{14} (2 μmoles per 8.1 × 10^5 c.p.m. per flask). Data represent the average of duplicate samples.

Addition	Specific activity	
	RNA adenine	RNA guanine
	c.p.m./μmole	*c.p.m./μmole*
Experiment I		
Control	261 ± 50	192 ± 11
Glucose (13 μmoles)	4,170 ± 290	3,640 ± 230
AIC (1 μmole)	2,880 ± 500	1,710 ± 80
Glucose (13 μmoles) + AIC (1 μmole)	8,850 ± 90	3,940 ± 50
Experiment II		
Control	183 ± 15	272 ± 20
Glucose (20 μmoles)	2,560 ± 20	3,250 ± 170
AIC (2 μmoles)	948 ± 63	1,140 ± 340
Glucose (20 μmoles) + AIC (2 μmoles)	4,070 ± 980	—
AIC-riboside (2 μmoles)	10,500 ± 400	3,390 ± 540
Glucose (20 μmoles) + AIC-riboside (2 μmoles)	11,600 ± 600	4,440 ± 400

TABLE IV

Effect of pyrimidine ribonucleosides and deoxyribonucleosides on incorporation of formate-C^{14} into RNA purines

Approximately 3 × 10^8 washed cells were suspended in Krebs-Ringer-phosphate buffer and incubated for 3 hours in the presence of formate-C^{14} (2 μmoles per 1.12 × 10^6 c.p.m. per flask). Data represent the averages of duplicate samples.

Addition	Specific activity	
	RNA adenine	RNA guanine
	c.p.m./μmole	*c.p.m./μmole*
Control	186 ± 25	175 ± 43
Glucose (20 μmoles)	1430 ± 60	1900 ± 520
Cytidine (2 μmoles)	1490 ± 50	1460 ± 40
Uridine (2 μmoles)	1060 ± 150	1140 ± 180
Deoxycytidine (2 μmoles)	155	173
Deoxyuridine (2 μmoles)	207 ± 73	121 ± 36

TABLE V

Effect of cytidine and deoxycytidine on incorporation of formate-C^{14}

2.29 × 10^8 cells were washed, suspended in 3.0 ml. of Krebs-Ringer-phosphate buffer and incubated for 3 hours at 37° in the presence of formate-C^{14} (2 μmoles per 1.05 × 10^6 c.p.m. per flask). Four flasks were combined for each analysis.

Addition	Specific activity in c.p.m./μmole							
	Acid-soluble			RNA		DNA		
	Adenine	Guanine	Thymine*	Adenine	Guanine	Adenine	Guanine	Thymine
Control	3,200	2,020	786	605	260	138	193	2320
Cytidine (2 μmoles)	30,800	18,900	2682	1930	975	377	340	3240
Deoxycytidine (2 μmoles)	2,740	1,920	7072	272	160	83	183	5020

* Total counts isolated after adding 2 μmoles of carrier thymine to acid-soluble fraction after the incubation and reisolation by paper chromatography.

TABLE VI

Effect of cytidine, AIC, and glucose on formate-C^{14} incorporation

In Experiment I 2.4 × 10^8 washed cells, suspended in 3 ml. of Krebs-Ringer-phosphate buffer, were incubated for 3 hours at 37° in the presence of formate-C^{14} (2 μmoles per 8.1 × 10^4 c.p.m. per flask). In Experiment II 1.8 × 10^8 washed cells, suspended in 3 ml. of Krebs-Ringer-phosphate buffer, were incubated for 3 hours at 37° in the presence of formate-C^{14} (2 μmoles per 2.7 × 10^5 c.p.m. per flask). Data represent the average of duplicate samples.

Addition	Specific activity	
	ASCE*	RNA
	c.p.m./unit†	*c.p.m./unit*†
Experiment I		
Control	37.6 ± 11.7	2.41 ± 0.60
Cytidine (2 μmoles)	118 ± 11	12.9 ± 0.3
AIC (2 μmoles)	90.2 ± 9.4	6.25 ± 0.08
Cytidine (2 μmoles) + AIC (2 μmoles)	396 ± 6	26.7 ± 1.9
Experiment II		
Control	122 ± 20	15.2 ± 2.2
Glucose (20 μmoles)	1216 ± 54	142 ± 3
Cytidine (2 μmoles)	1004 ± 61	87.7 ± 2.2
Glucose (20 μmoles) + cytidine (2 μmoles)	1206 ± 220	144 ± 6

* Acid-soluble charcoal eluate.

† Radioactivity divided by absorption at 260 mμ.

Prusoff *et al.* (3). The cytidine is probably converted to deoxycytidine which then either accepts formate-C^{14} directly or is first deaminated to deoxyuridine which accepts formate-C^{14} to form labeled thymidine (16–20).

A combination of cytidine and AIC stimulated formate-C^{14} incorporation into RNA more than either compound did alone, but a combination of cytidine and glucose was no more effective than either compound alone (Table VI). Thus it seems that both cytidine and glucose are utilized by the ascites tumor cells as a source of ribose-5-phosphate or PP-ribose-P for the synthesis of purine nucleotides. AIC serves as a source of purine ring precursors, and AIC-riboside provides a source of the purine ring and ribose simultaneously.

Although addition of glucose and AIC stimulated the incorporation of formate-C^{14} into purines, the incorporation of P^{32} into nucleic acids (3) and of formate-C^{14} into DNA thymine was not affected. It seems, therefore, that the amount of nucleic acid synthesis occurring in the system *in vitro* is adequately supported by endogenous preformed purine intermediates. Increasing purine synthesis *de novo* in the presence of formate-C^{14} could increase the specific activity of the endogenous purine pool and thus increase the amount of radioactivity entering nucleic acid purines without necessarily increasing the net synthesis of nucleic acid.

SUMMARY

The incorporation of formate-C^{14} into purines of the acid-soluble, ribonucleic acid, and deoxyribonucleic acid fractions by washed Ehrlich ascites tumor cells *in vitro* was found to be markedly stimulated by the addition of glucose, cytidine, uridine, or 4 (or 5)-amino-5 (or 4)-imidazolecarboxamide ribonucleoside. Ribose, ribose-5-phosphate, deoxycytidine, deoxyuridine, and various oxidizable substrates were ineffective. It has been postulated that the active compounds supply the system *in vitro* with a source of ribose phosphate or 5-phospho-α-ribosylpyrophosphate, necessary for the synthesis *de novo* of purine nucleosides.

Acknowledgments—The author is greatly indebted to Professor J. N. Davidson and Dr. R. Y. Thomson of the Biochemistry Department, University of Glasgow, for their advice and help and for the use of their facilities. The technical assistance of Mrs. Edith Seidman, Mrs. Jean Brattin, and Miss Rose Peterson is gratefully acknowledged.

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Biosynthesis of Deoxyribose in Intact *Escherichia coli**

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(Received for publication, June 13, 1958)

The synthesis of 2-deoxy-D-ribose has been investigated with C^{14} as a tracer to obtain information on the biosynthetic mechanism in intact *Escherichia coli*. Strain B of this organism was grown on labeled substrates, and the patterns of tracer found in the deoxypentose of the nucleic acids were evaluated in terms of specific proposed pathways.

Racker (1) has described a deoxyribose phosphate aldolase which forms deoxyribose-5-phosphate from acetaldehyde and glyceraldehyde-3-phosphate. Several groups (2, 3) have reported tracer data which suggest that pyrimidine ribonucleosides can be converted to the corresponding deoxyribonucleosides *in vivo*. Recently, Grossman and Hawkins (4) have demonstrated the conversion of uridine and cytidine to deoxyuridine and deoxycytidine by an extract of *Salmonella typhimurium*. Reichard (5) has shown that the reduction of ribose to deoxyribose can occur at the nucleotide level in extracts of chick embryos. Grossman (6) has also reported the conversion of ribonucleotide to deoxyribonucleotide and has shown that the enzymatic activity for this transformation increases markedly in *E. coli* upon infection with bacteriophage. Lanning and Cohen (7) have pointed out that decarboxylation of 2-keto-3-deoxy-6-phosphogluconate, an intermediate in the oxidation of glucose by *Pseudomonas saccharophila*, should yield deoxyribose-5-phosphate. No positive evidence seems to be available, however, for this mechanism. These pathways and their probable metabolic relationships are shown in Fig. 1.

The results obtained in this investigation do not indicate a major role for the deoxyribose phosphate aldolase in the synthesis of the deoxypentose in *E. coli* under these conditions. The data do, however, suggest that deoxyribose is derived directly from ribose or that both pentoses have a common precursor.

EXPERIMENTAL

Growth and Harvesting of Cells—When labeled glucose was the carbon source, growth conditions and harvesting procedures were the same as reported earlier (8). In the experiment with lactate-1-C^{14} as the carbon source, 0.8 per cent sodium lactate replaced the glucose in the medium. The remaining ingredients were not changed. In the latter case, the cells were harvested just before the end of the log phase of growth (14 hours after inoculation).

Isolation of Deoxyribonucleosides—The flow diagram (Diagram 1) illustrates the procedure for isolating the deoxyribonucleosides from the DNA of *E. coli*. The washed cells were treated with acetone and extracted with cold, 7 per cent $HClO_4$ (8). The combined sodium salts of RNA and DNA were then obtained from the residue by extraction with NaCl and precipitation with ethanol (9). RNA and DNA were separated by incubation in NaOH and subsequent acidification (8). The DNA was then degraded to the deoxyribonucleosides in the following manner. The DNA at a level of 3 μmoles of deoxyribose per ml. was incubated at 30° for 3 hours with 8 μg. of deoxyribonuclease[1] and 0.6 μmole of $MgSO_4$ per ml. The pH of the mixture was then adjusted to between 8 and 9, and 4 to 5 μmoles of alanine, pH 9.0, and 0.045 ml. of an aqueous extract of lyophilized venom (10 mg. per ml.) of *Crotalus adamanteus*[2] were added per ml. of "deoxyribonuclease" digest. The mixture was incubated for approximately 18 hours at 37° and then passed through a column of Dowex 1 ($HCOO^-$) to adsorb any remaining nucleotides.

The effluent which contained the deoxyribonucleosides was concentrated to dryness in a vacuum at room temperature and dissolved in 10 ml. of 0.02 M NH_4OOCH, pH 10.6 (adjusted with NH_4OH). This alkaline solution was passed through a column (resin bed dimensions were 0.8 cm. diameter × 19 cm. length) of Dowex 1 ($HCOO^-$) (10 per cent cross linkage) and the deoxyribonucleosides were eluted by the gradient technique of Hurlbert *et al.* (10) with the use of a mixing flask of a 100 ml. volume and a reservoir containing 500 ml. of 0.2 M NH_4OOCH, pH 10.6. The rate of inflow into the mixing flask equaled the rate of flow through the column. Fig. 2 illustrates the separation achieved. The formate was removed from the "thymidine" fraction in a vacuum at 55° in a rotating flask, and after addition of unlabeled carrier[3] the thymidine was recrystallized from hot water. The deoxycytidine and deoxyadenosine in the first fraction eluted from the column (after removal of formate) were separated by ascending (11) filter paper chromatography in a mixture of butanol-ethanol-H_2O-NH_4OH (12). In several cases these two deoxyribonucleosides were converted to thymidine which had a nonlabeled base with a nucleoside transdeoxyribosidase prepared from *Lactobacillus delbrueckii* as described by McNutt (13).

Isolation of Adenosine—Adenosine was isolated from the "RNA fraction" (Diagram 1). The ribonucleotides were hydrolyzed to the ribonucleosides with an acid prostatic phosphatase[4] in buffer at pH 5.5, and the latter were separated on a column of

* Preliminary reports of this investigation were presented at the meetings of the American Society of Biological Chemists in Chicago in April, 1957, and in Philadelphia in April, 1958. Partial support for this investigation has been provided by the Michigan Memorial-Phoenix Project (Project No. 112) of the University of Michigan and by the National Science Foundation (Grant No. G4514).

[1] Product of Worthington Biochemical Corporation, Freehold, New Jersey.

[2] Product of Ross Allen's Reptile Institute, Silver Springs, Florida.

[3] The thymidine used was obtained from the California Foundation for Biochemical Research, Los Angeles, California.

[4] Prepared according to Schmidt *et al.* (14).

Dowex 1 ($HCOO^-$) in the manner described above for the deoxyribonucleosides. A separation similar to that indicated for the deoxyribonucleosides in Fig. 2 was achieved except that uridine appeared where thymidine is shown. The adenosine and cytidine were separated by filter paper chromatography as described above for the respective deoxyribonucleosides. After elution from the filter paper the labeled adenosine was diluted with unlabeled adenosine and recrystallized from hot water.

Determinations of Radioactivity—The distribution of C^{14} in the deoxyribose moiety of thymidine was determined by fermentation with *Escherichia coli*, ATCC 9723, (15) and in adenosine by

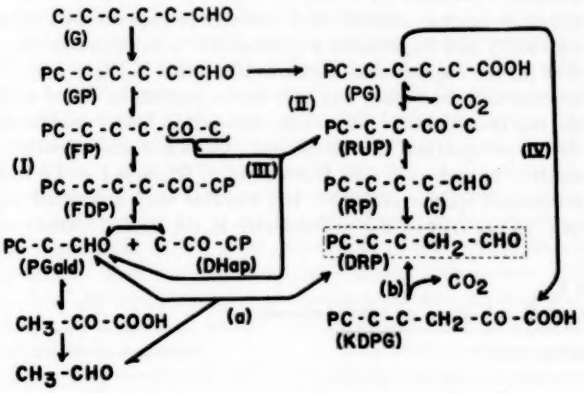

FIG. 1. Hypothetical pathways for the biosynthesis of deoxyribose and their probable metabolic relationships. Postulated synthetic pathways: (*a*) deoxyribose phosphate aldolase; (*b*) decarboxylation of 2-keto-3-deoxy-6-phosphogluconate; (*c*) reduction of ribose. I represents glycolysis; II, hexosemonophosphate oxidative pathway; III, "transketolase-transaldolase" enzyme systems; IV, oxidative pathway via 2-keto-3-deoxy-6-phosphogluconate. Abbreviations are G, glucose; GP, glucose-6-phosphate; FP, fructose-6-phosphate; FDP, fructose-1,6-diphosphate; PGald, 3-phosphoglyceraldehyde; DHap, dihydroxyacetone phosphate; PG, 6-phosphogluconate; RUP, ribulose-5-phosphate; RP, ribose-5-phosphate; DRP, deoxyribose-5phosphate; KDPG, 2-keto-3-deoxy-6-phosphogluconate.

DIAGRAM 1

Cells
- Acetone

Residue | Extract
- $HClO_4$, 0°

Residue | Extract (acid solubles)
- 10% NaCl

Extract (nucleic acids) | Residue (protein)
- 2 volumes EtOH

Precipitate (sodium nucleates) | Supernatant
- 0.5 N NaOH, 37°, 18 hours, Na^+ removed, acidified to pH 3

Precipitate (DNA) | Supernatant ("RNA fraction"—ribonucleotides)
- Deoxyribonuclease, snake venom
- Dowex 1 ($HCOO^-$) column

Effluent (deoxyribonucleosides) | Adsorbed (deoxyribonucleotides
- Dowex 1 ($HCOO^-$) column, gradient elution ($HCOONH_4$ at pH 10.6)

Eluate (Fraction A: deoxyadenosine, deoxycytidine*
Fraction B: thymidine
Fraction C: deoxyguanosine)

* Deoxyadenosine and deoxycytidine of Fraction A were separated by chromatography on filter paper with a mixture of butanol-ethanol-H_2O-NH_4OH (40:10:49:1 by volume) (12).

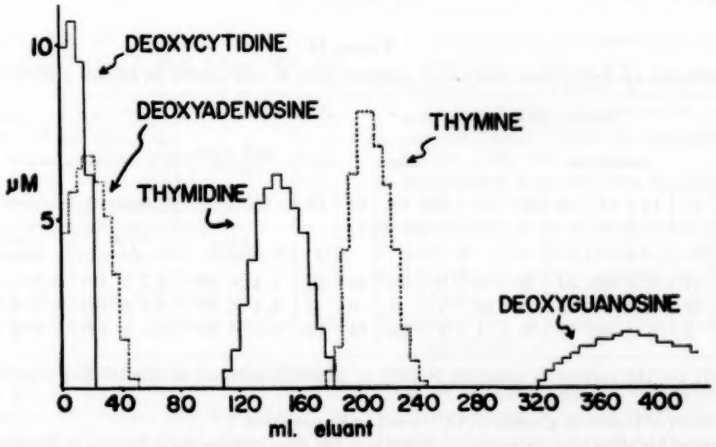

FIG. 2. Chromatographic separation of deoxyribonucleosides on Dowex 1 ($HCOO^-$) with gradient elution. Each component (run separately) at a level of 20 μmoles. For procedural details see the text.

fermentation with *Lactobacillus pentosus* (16). The total activity in the deoxyribose moiety of thymidine was determined either by preparation and total combustion of 4-aminopentanoic acid (15) or by subtracting the total activity in the isolated thymine from that in the undegraded deoxyribonucleoside (15). The specific activity of the ribose was determined by preparation (17) and combustion of furoic acid. Total combustion was carried out by the method of Van Slyke and Folch (18). Radioactivity was determined in a gas phase counter (19) to a standard counting error of 5 per cent or less.

Source of C^{14}-labeled Compounds—Glucose-1-C^{14}, glucose-2-C^{14}, and glucose-6-C^{14} were obtained from the National Bureau of Standards and from the Nuclear-Chicago Corporation. Lactic acid-1-C^{14} was also obtained from the Nuclear-Chicago Corporation. Glucose-3,4-C^{14} was prepared from the glycogen of rat liver after administration of $NaHC^{14}O_3$ (20).

RESULTS

Table I illustrates the patterns of C^{14} found in the deoxyribose when *E. coli* was grown on glucose-1-C^{14} (Experiment 1), glucose-2-C^{14} (Experiment 2), glucose-3,4-C^{14} (Experiment 3), or glucose-6-C^{14} (Experiment 4). The distributions of tracer found in the deoxyribose and ribose in Experiments 3 and 4 are compared in Table II. Also shown in Table II are the data obtained in regard to the two pentoses isolated from cells grown on lactate-1-C^{14}. In Experiment 1 the four deoxyribonucleosides were separately degraded and found to have essentially similar patterns of radioactivity.

DISCUSSION

If deoxyribose were synthesized by condensation of acetaldehyde and glyceraldehyde-3-phosphate catalyzed by deoxyribose phosphate aldolase (*Pathway a*, Fig. 1), Carbons 2 and 5 should be labeled primarily and similarly in Experiment 1 (glucose-1-C^{14}) and Experiment 4 (glucose-6-C^{14}) on the assumption that (*a*) the condensation occurs as illustrated in Fig. 1 and (*b*) the reactants are formed and have tracer patterns in accord with the reactions shown. The results obtained in both experiments do not substantiate this prediction. Carbon 2 was essentially inactive in each case. In Experiment 1, Carbons 1 and 5 had the largest specific activities, but together they accounted for only 13 per cent of the radioactivity in the labeled carbon of

TABLE I

C^{14} distribution in deoxyribose isolated from E. coli grown on labeled glucose

Experiment No.	Carbon source	Relative specific activity (RSA)*											Deoxyribose specific activity†		
		Glucose						Deoxyribose					RSA of 100 equals	Calculated‡	Found
		C6	C5	C4	C3	C2	C1	C5	C4	C3	C2	C1			
													c.p.m./ mmole C	*c.p.m./ mmole*	*c.p.m./ mmole*
1	Glucose-1-C^{14}	0	0	0	0	0	100	7	2	1	1	6	3.0×10^6	5.1×10^5	5.1×10^5
2	Glucose-2-C^{14}	0	0	0	0	100	0	8	30	4	15	42	5.0×10^5	5.0×10^5	5.0×10^5
3	Glucose-3,4-C^{14}§	3	2	100	91	1	3	17	7	103	54	10	1.4×10^5	2.7×10^5	2.8×10^5
4	Glucose-6-C^{14}	100	0	0	0	0	0	52	2	1	2	8	5.1×10^5	3.3×10^5	3.2×10^5

* RSA = (specific activity for the carbon in question × 100) ÷ (specific activity of the labeled glucose carbon given a value of 100).

† The values of C^{14} in deoxyribose have been corrected for dilutions by carrier. Carrier dilutions were 165-fold in Experiment 1, 371-fold in Experiment 2, 34-fold in Experiment 3, and 419-fold Experiment 4.

‡ Sum of RSA for the five carbons × specific activity corresponding to an RSA of 100 ÷ 100.

§ The pattern of labeling was determined by fermentation with *Leuconostoc mesenteroides* (20).

TABLE II

C^{14} distribution in deoxyribose and ribose isolated from E. coli grown on labeled glucose or lactate

Experiment No.	Carbon source	Relative specific activity (RSA)*										RSA of 100 equals†	Specific activities‡			
		Deoxyribose					Ribose						Deoxyribose		Ribose	
		C5	C4	C3	C2	C1	C5	C4	C3	C2	C1		Calculated§	Found	Calculated§	Found
												c.p.m./ mmole C	*c.p.m./ mmole*	*c.p.m./ mmole*	*c.p.m./ mmole*	*c.p.m./ mmole*
3	Glucose-3,4-C^{14}	17	7	103	54	10	0	0	93	42	12	1.4×10^5	2.7×10^5	2.8×10^5	1.9×10^5	1.9×10^5
4	Glucose-6-C^{14}	52	2	2	2	8	62	1	0	0	3	5.1×10^5	3.3×10^5	3.2×10^5	3.4×10^5	3.4×10^5
5	Lactate-1-C^{14}	2	3	62	12	21	1	1	69	15	10	1.4×10^6	1.3×10^6	1.3×10^6	1.3×10^6	1.2×10^6

* RSA = (Specific activity for the carbon in question × 100) ÷ (Specific activity of the labeled glucose or lactate carbon given a value of 100).

† See Table I for distribution of tracer in glucose-3,4-C^{14} used in Experiment 3.

‡ Values have been corrected for dilutions by carrier. Dilutions for deoxyribose were 34-fold in Experiment 3, 419-fold in Experiment 4, and 523-fold in Experiment 5; for ribose, 133-fold in Experiment 3, 31-fold in Experiment 4, and 500-fold in Experiment 5.

§ Sum of RSA for the five carbons × the specific activity corresponding to an RSA of 100 ÷ 100.

glucose. Lanning and Cohen (7), in similar experiments with glucose-1-C^{14}, had reported that the deoxypentose contained 20 to 26 per cent of the specific activity of the carbon source. These workers did not determine the distribution of the C^{14} in the molecule. In Experiment 4, Carbon 5 had a relative specific activity of 52 and Carbon 1, a value of 8. The results of these experiments, therefore, do not support the hypothesis that deoxyribose is synthesized by condensation of acetaldehyde and triose phosphate in *E. coli* under the conditions that obtained. It is interesting to note that deoxyribose phosphate aldolase can be routinely isolated from a strain of this organism (21). Possibly this enzyme is mainly concerned in the catabolism of the deoxypentose (*cf.* (15)) rather than its synthesis.

If deoxyribose were synthesized from ribose by direct reduction at Carbon 2 (*Pathway c*, Fig. 1) or through an intermediate common to both sugars, the patterns of labeling in the two pentoses should be similar in all cases. The distribution of tracer in the deoxyribose in each of the experiments shown in Table I is qualitatively the same as that previously reported (8) for ribose obtained under similar experimental conditions. Even closer agreement is seen when the tracer patterns of the two carbohydrates are determined in the same experiment (Table II). Bagatell *et al.* (22) have obtained comparable results with acetate-1-C^{14} in *E. coli* R2 adapted to grow on acetate as the sole carbon source. Although these data support the hypothesis that deoxyribose originates from ribose or that both arise from a common precursor, they do not rule out the possibility that a currently unknown pathway may also explain these results and be the actual mechanism for the biosynthesis of deoxyribose in *E. coli*. It should further be emphasized that certain aspects of the data are not explained by the "direct reduction" hypothesis. For example, the mechanism for the incorporation of C^{14} in Carbon 5 of deoxyribose in Experiment 3 (glucose-3,4-C^{14}) is not indicated. Carbon 5 of ribose was not labeled in that experiment.

It is likely that ribose and deoxyribose would also have similar patterns of C^{14} if the latter were synthesized by decarboxylation of 2-keto-3-deoxy-6-phosphogluconate (*Pathway b*, Fig. 1), since 6-phosphogluconate would be a common intermediate in the formation of the two compounds. One could expect similar patterns in the two carbohydrates regardless of the mechanism for the synthesis of ribose, because ribose-5-phosphate can be converted to 6-phosphogluconate by the addition of CO_2 to ribulose-5-phosphate (23). There seems to be no evidence in the literature, however, for the existence of an enzyme which decarboxylates 2-keto-3-deoxy-6-phosphogluconate.

These findings in *E. coli* do not obviate the possibility that the synthesis of deoxyribose occurs by a different mechanism in other cells and tissues. For example, ribose[5] and deoxyribose (24) have different patterns of tracer when isolated from regenerating rat liver after injection of C^{14}-labeled glycine, and the two carbons of labeled acetate are incorporated in different ratios into the two sugars when slices of regenerating rat liver are incubated with this precursor (25). The radioactivity in deoxyribose was also different[6] from that in ribose (26) when these compounds were isolated from the normal livers of rats which had received $NaHC^{14}O_3$.

It seems probable that the mechanism for the synthesis of ribose can vary in *E. coli*, since the distribution of C^{14} in the pentose was different in the current work (Experiment 3) from the distribution in earlier investigations (8) when glucose-3,4-C^{14} was the carbon source.

SUMMARY

The biosynthesis of deoxyribose has been studied in intact *Escherichia coli* by means of the tracer technique. Thymidine and adenosine were isolated from cells grown on specifically C^{14}-labeled glucose or lactate as the sole carbon sources and were degraded to determine the radioactivity in each carbon of the "pentose" moieties of both nucleosides.

The patterns of C^{14} found in the deoxyribose indicate that, when this sugar is synthesized from glucose, Carbon 1 of the hexose is lost. A comparison of the distributions of tracer in the deoxypentose with those found in ribose suggests that deoxyribose arises from ribose or that both have a common precursor. Neither of these explanations, however, accounts for certain aspects of the data.

Acknowledgment—The authors are grateful to Dr. Arthur C. Curtis for his interest in and support of this investigation.

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[5] W. W. Shreeve, personal communication.

[6] The patterns of tracer in the deoxyribose and ribose were determined in separate but similar experiments. Only Carbons 1 and 3 were labeled in the deoxyribose. The ratio of specific activities of Carbon 1 to that of 3 was 0.62.

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Bacterial Degradation of Deoxyribose-C^{14}*

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(Received for publication, June 13, 1958)

The anaerobic metabolism of thymidine-C^{14} by resting cells of *Escherichia coli*, ATCC 9723, has been studied to ascertain whether this organism could be used to determine the radioactivity in each carbon of the deoxypentose moiety. A technique for obtaining the pattern of labeling in the sugar was necessary in connection with a tracer study of the biosynthesis of 2-deoxy-D-ribose in intact cells.

Bacterial fermentations as a procedure for degradation of carbohydrates are particularly advantageous when direct chemical attack fails to yield each carbon separately or would necessitate considerable quantities of material for complete degradation (*cf.* (1)). To be useful for this purpose, however, a fermentation must satisfy at least the following two requirements. (*a*) The sugar must be cleaved into products which can be further conveniently degraded chemically to give each carbon separately. (*b*) Each carbon in these products must arise from a single carbon in the carbohydrate without significant contribution from the other carbons (*i.e.* without "cross contamination").

Resting cells of *E. coli*, ATCC 9723, anaerobically cleave thymidine (2) and thus liberate the free base and convert the deoxyribose moiety into ethanol, formate (or CO_2 and H_2), and acetate (3). Approximately 1 mole of each of the products is formed per mole of deoxyribonucleoside which disappears (4), and each of the products can easily be further degraded chemically into its separate carbons (*cf.* (1)).

The mechanism for this fermentation postulated by Hoffman and Lampen (3) would result in an over-all reaction carried out by anaerobically grown cells as presented in Scheme I. The numbers refer to the carbons in the deoxyribose from which the carbons in the products arise. In order to evaluate the hypothesis, aliquots of the same sample of thymidine-C^{14} were degraded separately by the bacterial procedure and by chemical means exclusively, and the distributions of tracer indicated by the two methods were compared. The data obtained support the proposed concept of the fermentation and indicate no significant "cross contamination" of carbon in the products.

EXPERIMENTAL

Preparation of Bacteria—Cells of *E. coli*, ATCC 9723, were grown anaerobically on Medium 1 as described by Hoffman and Lampen (3) for use in the degradation of deoxyribonucleosides. The cells were maintained on slants of the same medium plus 2 per cent agar. When the cells were to be used for degradation, they were harvested by centrifugation, washed once with 0.05 M $NaHCO_3$ and twice with distilled H_2O and prepared as a 20 per cent suspension (1 gm., wet weight, plus 4 ml.) in 0.9 per cent NaCl.

Incubation Procedure[1]—The bacterial degradations were carried out in 125-ml. Warburg respirometer flasks (with 2 side arms) with shaking in a water bath at 37°. The reaction mixture consisted of 2 mmoles of phosphate buffer at pH 7.5, 0.5 mmole of deoxyribonucleoside, and 7 ml. of cell suspension in a final volume of 30 ml. The CO_2 formed in the reaction was continuously removed from the flask (through a "vented" stopper on one of the side arms) in a gentle stream of N_2 and recovered by passage through a "coil" trap immersed in liquid N_2 (1). 1 ml. of 10 N H_2SO_4 initially present in a side arm was added at the end of the procedure to release any CO_2 remaining in the solution. The degradation of 0.5 mmole of deoxyribonucleoside usually required 2 hours. A flask without substrate was included in each experiment to determine the endogenous formation of products. Table I illustrates the yields of products generally obtained in this procedure.

Recovery and Chemical Degradation of Bacterial Products[1]—The cells were removed by centrifugation and the ethanol was recovered by distilling three-fourths of the supernatant solution after neutralization with NaOH to phenol red. The residue was then acidified to Congo red with H_2SO_4, and the acetic acid (and any formic acid) was steam distilled. The ethanol was oxidized to acetic acid. The two samples of acetate were separately purified and degraded to $BaCO_3$ and CHI_3 by pyrolysis of barium acetate and treatment of the resulting acetone with NaOI. The $BaCO_3$ and CHI_3 would represent the carbinol and methyl carbons of the original ethanol and the carboxyl and methyl carbons of the original acetate, respectively. Scheme I indicates the assumed origin of each carbon of the ethanol and acetate as well as of the CO_2.

Chemical Degradation of Thymidine—Thymidine was degraded chemically by the reactions illustrated in Scheme II.

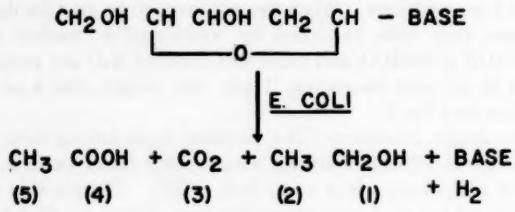
Thymidine was hydrolyzed and converted to thymine and levulinic acid by refluxing 1 mmole of the deoxyribonucleoside in 4 ml. of 10 N H_2SO_4 for 5 hours followed by standing at room temperature for 24 hours. The impure thymine which precipitated was removed by filtration, and the levulinic acid was recovered from the filtrate by continuous ether extraction for approximately 48 hours. More thymine crystallized out of the ether extract. Thymine (from both sources) was purified by

* This investigation has been supported by grants from the Michigan Memorial-Phoenix Project (Project No. 112) of the University of Michigan and from the National Science Foundation (Grant No. G4514). A preliminary report was presented to the Society of American Bacteriologists in Chicago in April, 1958.

[1] For a detailed description of the technique see the examples given by Bernstein and Wood (1).

sublimation in a vacuum. The levulinic acid in the ether extract was *carefully* neutralized with NaOH to phenol red, concentrated into about 1 ml., and further purified by partition chromatography on a column of Celite No. 535 as described for acetate (1). Levulinic acid was eluted from the column with 100 per cent $CHCl_3$.

The levulinic acid was degraded by several techniques. One procedure involved treatment with NaOI which yielded CHI_3 (carbon 5) and succinic acid (carbons 1, 2, 3, and 4). To 1 mmole of levulinic acid in approximately 50 ml. of H_2O were added 25 ml. of cold 1 N NaOH and then 72 ml. of cold 0.1 N I_2, added dropwise with stirring. The reaction mixture was kept at 0° during the procedure and for 2 to 14 hours thereafter. The iodine was then treated with $NaAsO_2$ (1), and the CHI_3 was removed by filtration and oxidized to CO_2 (1). The succinic acid in the filtrate was recovered by continuous ether extraction and purified by partition chromatography on Celite No. 535 (5), and by sublimation in a vacuum (6). The melting point of the isolated and an authentic sample was 185°; uncorrected. The acid was converted to its anhydride and decarboxylated by the Schmidt reaction[2] as described by Phares and Long (7) to form CO_2 (carbons 1 and 4 of the levulinic acid and deoxyribose) and ethylenediamine (carbons 2 and 3).[3] The diamine was isolated by vacuum distillation and was oxidized to CO_2 (8).



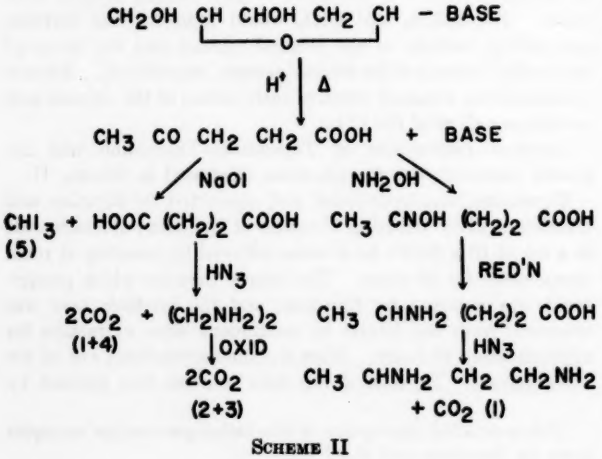
SCHEME I

TABLE I

*Analytical data on bacterial degradation of thymidine**

Procedure	Thymidine C^{14}	Endogenous
	mmole	*mmole*
Thymidine submitted to fermentation	0.593	0
CO_2 recovered during fermentation (C3)	0.318	0.031
Acetate isolated from Celite column (C4, 5)	0.502	0.050
Ethanol as acetate isolated from Celite column (C1, 2)	0.586	0.046
CO_2 from $BaCO_3$ (C1)	0.120	
CO_2 from CHI_3 (C2)	0.085	
CO_2 from $BaCO_3$ (C4)	0.141	
CO_2 from CHI_3 (C5)	0.080	

* Data from Experiment 4, Table III. See the text for details of the procedures.



SCHEME II

In order to obtain a value for the specific activity of carbon 1, levulinic acid was converted to 4-aminopentanoic acid by means of reduction of the oxime and then the amino acid was decarboxylated by the Schmidt reaction. The following procedures were used (9). To 1 mmole of hydroxylamine sulfate in 10 ml. of 0.1 M NaOH were added 10 ml. of 0.1 M sodium levulinate. The mixture was kept at room temperature for 36 hours, acidified with H_2SO_4 to Congo red, and the oxime recovered by continuous ether extraction for 18 hours. After removal of the ether, the compound was dried in a vacuum and recrystallized from methanol to give a product with an uncorrected melting point of 96° (recorded, 95–96° (9)). The oxime was usually obtained in 75 to 85 per cent yields.

The oxime was reduced to 4-aminopentanoic acid (10) with aluminum amalgam. Aluminum foil[4] was washed 3 times with 50 ml. each of ether, acetone, and distilled H_2O, exposed for exactly 3 minutes to 5 ml. of 2 per cent $HgCl_2$, and washed quickly 2 times with 5 ml. of 80 per cent ethanol. After the last wash 1 mmole of oxime in 5 ml. of 80 per cent ethanol was added, and the reaction flask (equipped with a mercury, pressure-release valve) was stoppered and shaken for 5 hours at room temperature and then allowed to stand overnight. The mixture was filtered and the residue was washed well with H_2O. The combined filtrates were concentrated to dryness in a vacuum without heating, and the 4-aminopentanoic acid was crystallized from a mixture of water, ethanol, and ether. The recrystallized amino acid had a decomposition point of 190–192°, uncorrected. Cornforth and Popják (10) report a value of "about 200°" for the solvated crystal.

Application of the Schmidt reaction as described above[2] to the 4-aminopentanoic acid yielded 80 per cent or more of CO_2 from the carboxyl group presumed to represent carbon 1 of the deoxypentose. The resulting diamine was not recovered.

Table II illustrates the yields obtained by the above chemical procedures.

Conversion of Cytidine Deoxyriboside to Thymidine—The thymidine sample of Experiment 4 (Table III) was obtained by con-

[2] CO_2 was trapped as a gas, measured in a vacuum system and counted in silver plated tubes. During early use of this reaction, we found that another gaseous material accompanied the CO_2 and reacted with the mercury of the vacuum system and the silver of the counter tubes. This difficulty was eliminated by first passing the CO_2 through a tube containing platinum gauze at 1000° and then a trap immersed in a Cellosolve-Dry Ice mixture.

[3] The yield of CO_2 was never as high as that obtained by Phares, and β-alanine could be isolated to account for the unrecovered CO_2.

[4] Catalogue No. 1-213, Fisher Scientific Company, Pittsburgh, Pennsylvania.

version from deoxycytidine-C^{14} with the use of unlabeled thymine and an extract of *Lactobacillus delbrueckii*, ATCC 9649, as described by McNutt (11).

Isotopic Analyses—Radioactivity was determined by counting CO_2 in a gas phase proportional counter (12). Specific activities of thymine and thymidine were determined by combustion to CO_2 (8). All samples were counted to a standard error of 5 per cent or less except as noted in Table III.

Source of Samples—All samples of thymidine-C^{14} were obtained from *E. coli*, strain B, grown on glucose-1-C^{14} (Experiment 1), glucose-2-C^{14} (Experiment 2), glucose-6-C^{14} (Experiment 3), or lactic acid-1-C^{14} (Experiment 4) (13).

TABLE II

*Analytical data on chemical degradation of thymidine**

Procedure	Yield
	mmoles
Thymidine submitted to hydrolysis	1.91
Levulinic acid isolated from Celite column	1.76
Degradation via succinic acid	
Levulinic acid submitted to oxidation by hypoiodite	0.902
CO_2 from CHI_3 (C5)	0.375
Succinic acid isolated from Celite column and submitted to decarboxylation	0.258
CO_2 from succinic acid (C1, 4)	0.181
CO_2 from ethylenediamine (C2, 3)	0.129
Degradation via 4-aminopentanoic acid	
Levulinic acid used in preparation of levulinic oxime	0.801
4-Aminopentanoic acid isolated upon reduction of levulinic oxime	0.196
CO_2 from 4-aminopentanoic acid (C1)	0.203

* Data from Experiment 4, Table III. For details of procedures, see the text.

TABLE III

*Distribution of C^{14} found in deoxyribose of thymidine by bacterial and chemical procedures**

Experiment No.	Thymidine†	Bacterial procedure						Chemical procedure						
		C5	C4	C3	C2	C1	Thymine	4-Aminopentanoic acid (C1–5)	Succinic acid (C1–4)	C5	$\frac{C1+4}{2}$	$\frac{C2+3}{2}$	C1	Thymine
1	3000	275	161	12	185	362	1915	984						1850
2	960	0	195	7	95	312				7	244	57		
3	2240	638	30	20	43	103	1340			671				
4	1120	38	14	661	126	223			1078	38	119	416	218	0

* Data are in counts per minute per mmole of carbon for the fragments and per mmole of compound in the other cases. All isotopic data obtained by fermentation shown as corrected for endogenous dilution. All values, except those less than 10 counts per minute, were determined to a standard counting error of 5 per cent or less.

† Thymidine samples were isolated from *Escherichia coli*, strain B, grown on glucose-1-C^{14} (Experiment 1), glucose-2-C^{14} (Experiment 2), glucose-6-C^{14} (Experiment 3), and lactic acid-1-C^{14} (Experiment 4) (13). The sample of thymidine in Experiment 4 was prepared from cytidine-C^{14} and thymine-C^{13} by the procedure of McNutt (11).

RESULTS

Table III presents the data obtained when aliquots of the same sample of thymidine-C^{14} were submitted to bacterial and chemical procedures. In Experiment 1 a total of 995 c.p.m. per mmole was found in the deoxyribose by bacterial fermentation and 984 by total combustion of 4-aminopentanoic acid. In Experiment 2 609 c.p.m. were observed in the deoxypentose by fermentation and by chemical degradation. The specific activities of the carbons of the deoxypentose according to the two techniques were as follows. Carbons 1 and 4 averaged 254 by the bacterial procedure and 244 by chemical determination, carbons 2 and 3 similarly were found to be 51 and 57, and carbon 5 had values of 0 and 7. In Experiment 3 carbon 5 was shown to contain 638 and 671 c.p.m. per mmole of carbon by the bacterial and chemical procedures, respectively. The total activity of the deoxypentose moiety in Experiment 4 was 1062 by fermentation and 1120 and 1110 by the two chemical procedures. Carbons 1 and 4 averaged 119 both by fermentation and decarboxylation of succinic acid, and carbons 2 and 3 averaged 394 and 416, respectively, by the two techniques. The bacterial and chemical methods indicated 223 and 218, respectively, as the values for carbon 1.

DISCUSSION

The data in Table III indicate that *E. coli*, ATCC 9723, degrades the deoxypentose molecule into three parts consisting of carbons 1 and 2 (ethanol), carbon 3 (CO_2), and carbons 4 and 5 (acetate). These results support the proposal by Hoffman and Lampen (3) as to the mechanism for the fermentation. Accordingly, deoxypentose phosphate would be initially split into acetaldehyde (carbons 1 and 2) and glyceraldehyde phosphate (carbons 3, 4, and 5) possibly by means of the deoxyribose phosphate aldolase described by Racker (14). The acetaldehyde would then be reduced to ethanol and the glyceraldehyde phosphate, oxidized to pyruvate. The pyruvate would be split into formate and acetate and the formate, oxidized to CO_2 with the evolution of H_2. In addition, the data show that this fermentation provides a convenient means of determining the distribution of radioactive carbon in the deoxypentose of nucleic acid and thus makes it possible to study the biosynthesis of this sugar in the intact cell.

SUMMARY

Data obtained from chemical and bacterial degradation of deoxyribose-C^{14} (as thymidine) indicate that *Escherichia coli*, ATCC 9723, cleaves deoxypentose into three parts which arise as follows: the carbinol and methyl carbons of ethanol from carbons 1 and 2 of the sugar; CO_2 from carbon 3; and the carboxyl and methyl carbons of acetate from carbons 4 and 5, in that order. The validity of this fermentation process as a means to determine the distribution of a tracer in deoxyribose has been described.

Acknowledgment—The authors are grateful for the support of this investigation provided by Dr. Arthur C. Curtis.

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The Reaction of Quaternary Pyridine Derivatives with Imidazoles*

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(Received for publication, June 16, 1958)

Diphosphopyridine nucleotide reacts chemically with a number of reagents, including alkali (1), cyanide (2, 3), bisulfite ions (2, 3), dihydroxyacetone (4), dithionite ions (5), hydroxylamine (6), mercaptans (7), and aromatic amines (8). All of these reagents are nucleophilic agents which react on a positive center. It is generally assumed that these reactions involve the addition of the nucleophilic agent to the position of the nicotinamide moiety where the hydrogen addition occurs during enzymatic or chemical reduction, *i.e.* the para position (9). This assumption has proved valid for the cyanide (10) and dithionite (5) addition reactions.

In one instance such an addition reaction seems to exist naturally. The binding of DPN to triosephosphate dehydrogenase from yeast or rabbit skeletal muscle results in an increased absorption in the region around 360 mμ (11). This results in a spectrum of the enzyme-DPN complex which is similar to that of the addition complexes enumerated above. The analogue of DPN, acetylpyridine-DPN, shows this effect in a much more outstanding manner (12). It has been assumed (11, 7) that this spectral band represents a mercaptan addition to the DPN. However, two other groupings on the protein could conceivably interact with DPN and thus be responsible for such a spectrum. These are the imidazole group of histidine and the phenolic group of tyrosine.

The present paper describes the interaction which can exist between DPN and imidazoles.

EXPERIMENTAL

Pyridine Derivatives—DPN, acetylpyridine-DPN (13, 14), and pyridinealdehyde-DPN (13, 15) were products of the Pabst Laboratories. The latter two nucleotides were generously supplied by Dr. Nathan O. Kaplan.

The *N*-methyl pyridinium derivatives were prepared from the corresponding free bases by reaction with methyl iodide. The following were synthesized:[1] *N'*-methyl-nicotinamide iodide, m.p. 201–202° (reported: 202.7–203.4° (16)); *N*-methyl-3-acetylpyridinium iodide m.p. 155–156° (reported: 154.5–155.2° (17) and 163–164° (18)); *N*-methyl-3-formyl-pyridinium iodide, m.p. 165–167° (reported: 164.5–166° (17), and 173° (18)); *N'*-methyl-3-carboxime-pyridinium iodide, m.p. 149–151° (reported: 154–155° (18)); and *N'*-methyl-2-carboxime-pyridinium iodide, m.p. 224–226° decomposition point (reported: 224–225° (18)).

The *N*-benzyl pyridinium derivatives were prepared, in essentially quantitative yields, by refluxing the pyridine base in either dry benzene or absolute ethanol with benzyl bromide. The compounds were recrystallized from ethanol or methanol. The following were prepared:

1. *N'*-benzyl-3-carboxamide-pyridinium bromide:[2] fine white needles, m.p. 210–211°.

$C_{13}H_{13}N_2OBr$ (294.2)

Calculated: N 9.55
Found: N 9.62

2. *N'*-benzyl-3-carboxime-pyridinium bromide: very fine white needles, m.p. 184–187°.

$C_{13}H_{13}N_2OBr$ (2.94.2)

Calculated: N 9.55
Found: N 9.47

3. *N'*-benzyl-2-carboxime-pyridinium bromide: fine white, glistening platelets, m.p. 213–214° decomposition point.

$C_{13}H_{13}N_2OBr$ (294.2)

Calculated: N 9.55
Found: N 9.43

4. *N*-benzyl-3-acetyl-pyridinium bromide: long white needles, m.p. 195–197°.

$C_{14}H_{14}NOBr$ (291.2)

Calculated: N 4.79
Found: N 4.76

N-benzyl-3-formyl-pyridinium bromide was an oil which could not be induced to crystallize. Previously the chlorides of *N'*-benzyl nicotinamide (19) and *N*-benzyl-3-acetylpyridine (20) have been reported.

The free pyridine bases were commercial preparations, with the exception of the oximes, which were prepared from the corresponding aldehydes (18).

Imidazole Derivatives—Imidazole, benzimidazole, 2-methyl benzimidazole, histamine, histidine, and 1-methyl histidine were commercial preparations. All were used without further purification, with the exception of 2-methyl benzimidazole which was recrystallized several times from water.

2-Imidazolone was prepared with 2-imidazolone-4-carboxylic acid by the method of Hilbert (21, 22). The product had a

* This investigation was supported in part by an institutional grant from the American Cancer Society.

† Investigator in the Howard Hughes Medical Institute.

[1] All melting points were taken on a Fischer-Jones melting-point apparatus and are uncorrected.

[2] Elementary analyses were performed by the Clark Microanalytical Laboratory, Urbana, Illinois.

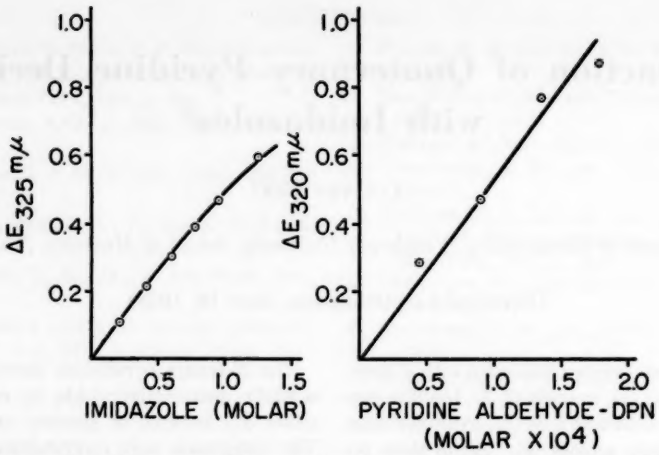

FIG. 1. The effect of imidazole and pyridine nucleotide concentration on the addition complex. *Left*, 0.1 M phosphate buffer, pH 10.0; imidazole concentration as indicated, and acetylpyridine-DPN, 500 μg. The final volume is 3.0 ml. *Right*, 0.1 M phosphate buffer, pH 10.0. The imidazole concentration is 0.7 M; pyridinealdehyde-DPN as indicated in a final volume of 3.0 ml.

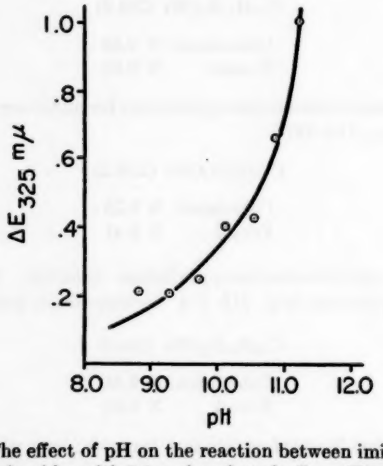

FIG. 2. The effect of pH on the reaction between imidazole and pyridine nucleotides with 0.1 M phosphate buffer, pH as indicated, 0.6 M imidazole, and 500 μg. of pyridinealdehyde-DPN. The observed extinctions are corrected for the effect of alkali on pyridinealdehyde-DPN. The final volume is 3.0 ml.

melting point of 249.5–250° (reported: 251–252° (23)). 2-Methyl imidazole was prepared from tartaric acid dinitrate (23), acetaldehyde and ammonia (24), m.p. 132–134° (reported: 134–136° (24)). The picrate was prepared from the free base: m.p. 206° (reported: 213° (25)). Alternately 2-methyl imidazole was prepared in low yield (10 per cent) as described for 2,4,5-trimethyl imidazole (26) by substitution of glyoxal for diacetyl.

4(5)-Tetrahydroxybutyl imidazole and carnosine were generous gifts of Dr. William J. Darby. Anserine and part of the 1-methyl histidine used were gifts from Dr. H. V. Aposhian.

Enzymes—Crystalline yeast alcohol dehydrogenase and crystalline rabbit skeletal muscle lactic dehydrogenase were purchased from the Worthington Biochemical Corporation. Crystalline α-glycerolphosphate dehydrogenase was prepared from rabbit skeletal muscle by the method of von Beisenherz *et al.* (27). *Neurospora* DPNase (28) was a generous gift of Dr. Anthony San Pietro. Pig brain DPNase was prepared by the method of Zatman *et al.* (29). As a source of rat liver DPNase, a 1:10 homogenate in 0.1 M phosphate buffer, pH 6.8, was used.

Determinations—The concentration of pyridine nucleotides was determined by the reaction resulting from the addition of cyanide (3), or occasionally by alcohol dehydrogenase assay. The following molar extinction coefficients were used: for DPN, 6.3×10^3 (30); for acetylpyridine-DPN, 7.8×10^3 (13), and for pyridinealdehyde-DPN, 7.0×10^3 (13, 15), all at their absorption maximum in the 300 to 400 mμ region.

Concentration of imidazole was determined by titrating an aliquot with standard acid, of inorganic phosphate by the method of Lowry and Lopez (31), and of total phosphate by the method of Fiske and SubbaRow (32). Ribose was estimated by the orcinol procedure (33) with the use of 5′-adenylic acid as a standard, and nicotinic acid was estimated by microbiological assay (34) with *Lactobacillus arabinosus*. The Pauly diazo reaction was performed by the method of McPherson (35). For the cyanogen bromide reaction the method of Das and Ghosh was used (36).

RESULTS

Reaction of Imidazole with Pyridine Nucleotides

When DPN or DPN analogues were added to a solution containing imidazole, an increase in ultraviolet absorption occurred in the region of 300 to 400 mμ. The increase in absorption is dependent on the concentration of imidazole, the concentration of the pyridine nucleotide, and the hydrogen ion concentration. Figs. 1 and 2 illustrate these points for representative reaction mixtures.

As is the case with other addition reactions, acetylpyridine-DPN and pyridinealdehyde-DPN show a much more favorable addition reaction than does DPN.

Specificity of Reaction—This addition reaction is given by a variety of imidazole derivatives. Although for a number of those tested only indications of reactions were observed because of the limited amounts available, conclusive spectral evidence could be obtained with imidazole, 2-methyl imidazole, histamine,

histidine, and 4(5)-tetrahydroxylbutyl imidazole. Benzimidazole and 2-methylbenzimidazole also showed evidence of the addition, but the complex seemed to be much weaker. Most reactions could be especially well observed in methanol as solvent, which obviates the requirement for a highly alkaline solution. 1-Methyl histidine and 2-imidazolone failed to interact under any of the conditions tried.[3]

The addition reaction is also not limited to pyridine nucleotides. All quaternary pyridine derivatives tested show the reaction. Some of the absorption maxima observed when different compounds interact with imidazole derivatives are shown in Table I. A representative spectrum of an addition complex, that of the imidazole-acetylpyridine-DPN complex, is shown in Fig. 3. It is interesting to note that again *N*-methylpyridinium derivatives have absorption maxima for their complexes approximately 10 mμ higher than those of the nucleotides.

As was shown for previous addition reactions, the quaternary nitrogen was essential for the interaction between pyridine and imidazole derivatives; pyridinealdehyde, acetylpyridine, or nicotinamide fail to show any evidence for addition, even at high base concentration.

Extinction Coefficients of Reaction Products—When the molar extinction coefficients were measured at the maximal absorption for the imidazole-pyridine nucleotide complexes the following results were obtained: for the imidazole-DPN complex, 6.8×10^3; for the imidazole-acetylpyridine-DPN complex, 1.18×10^4; and for the imidazole-pyridinealdehyde-DPN complex, 1.39×10^4. These extinction values are significantly higher than those observed for any other addition complex so far described.

Equilibrium Constant of Reaction—With the use of the above extinction coefficients the dissociation constant of the equilibrium, imidazole + pyridine nucleotide $\rightleftharpoons$ complex + H^+, could be determined. The complexes are weak, therefore some variation was observed, but the order of magnitude is correct. The following values were obtained: for DPN, $1.23 \pm 0.6 \times 10^{-12}$; for acetylpyridine-DPN, $2.8 \pm 1.2 \times 10^{-11}$; and for pyridinealdehyde-DPN, $1.2 \pm 0.02 \times 10^{-11}$. The average figures with their standard deviations are given. These figures indicate that again the order of ease of addition is: DPN, the least favorable; pyridinealdehyde-DPN, the most favorable; and acetylpyridine-DPN, intermediate.

The *N*-methyl derivatives are much less favorable in their addition than are the nucleotides, whereas the *N*-benzyl derivatives seem to be intermediate between these extremes.

Effect of Ferricyanide on Complexes—Since imidazole is relatively stable to oxidation, it was feasible to prepare the oxidized analogues of the imidazole addition complexes by treatment with ferricyanide. This is entirely analogous to the synthesis of the oxidized dihydroxyacetone addition complexes (37, 38).

The reaction could readily be followed by measurement of the ferri-ferrocyanide formed after addition of aliquots taken from the reaction mixture to a solution containing ferric sulfate. Such an experiment is shown in Fig. 4. As can be seen, it was not essential to free the complex from excess imidazole. By this method it has been possible to isolate oxidized imidazole complexes in pure form.

[3] Previously a complex between pyridinealdehyde-DPN and ergothioneine had been reported (7). On the basis of evidence in this paper, this could represent an imidazole-nucleotide complex rather than a mercaptan-nucleotide complex. However, in view of the more favorable addition of mercaptan, the original interpretation seems the more likely.

TABLE I

Observed absorption maxima for reaction between pyridinium derivatives and imidazoles

The absorption spectra were determined for the histidine and histamine solutions in 0.1 M phosphate buffer and for the imidazole derivative in 0.2 M phosphate buffer, and adjustments were made to the required pH. After the spectrum was ascertained against phosphate as a blank, the pyridine base was added, usually 500 μg. for the nucleotides and 100 μg. for the other derivatives. The bases were added to both the blank and experimental cuvettes. pH control was found to be of the utmost importance. Imidazole additions were usually determined by adding the pyridine derivative to 6 M imidazole solutions.

Pyridine derivative		Imidazole derivative		
Nitrogen substituent	Other ring substituent	Imidazole	Histidine	Histamine
		mμ	mμ	mμ
Methyl	3-Carboxamide	315	—*	
	3-Acetyl	335	340	
	3-Formyl	330	335	
	3-Carboxime	300	—*	
	2-Carboxime	350	—†	
Benzyl	3-Carboxamide	315	—*	
	3-Acetyl	335	340	
	3-Carboxime	300	—*	
	2-Carboxime	350	—†	
ADPR‡	3-Carboxamide	305		
	3-Acetyl	325	330	310
	3-Formyl	325	325	—§
ATPR‡	3-Carboxamide	305		

* The reaction is too unfavorable to observe the addition complex.

† The alkali shift in spectrum completely obscures the addition reaction.

‡ ADPR stands for the adenosine diphosphate ribose moiety of DPN, and ATPR stands for the adenosine diphosphate ribose phosphate moiety of TPN.

§ In phosphate buffer histamine seems to destroy pyridinealdehyde-DPN. On mixing of pyridinealdehyde-DPN and histamine in phosphate buffer, pH 10.5, an initial sharp peak appears which subsequently disappears rapidly.

Preparation of 1-Methyl-3-acetyl-4-(1-imidazolyl)-pyridinium Reineckate—1.26 gm. (5 mmoles) of *N*-methyl-3-acetyl-pyridinium iodide, 1.36 gm. (20 mmoles) of imidazole, and 2.0 gm. (6 mmoles) of potassium ferricyanide were dissolved in 30 ml. of distilled water. The mixture was cooled in ice, stirred by a stream of nitrogen, and kept slightly alkaline by the cautious dropwise addition of 0.40 gm. (7 mmoles) of potassium hydroxide dissolved in 10 ml. of distilled water. Great care was taken to avoid a local excess of base. When the reaction was complete, as was determined by ferrocyanide formation (2.5 hours), 10 ml. of a saturated solution of Reinecke salt in methanol were added. The mixture was allowed to stand for 1 additional hour in the cold in order to complete crystallization. The crystals were filtered off, and washed first with small portions of ice-cold

water and then with *n*-propanol. The isolated salt was recrystallized by suspension in a small amount of hot water to which acetone was added until just dissolved. The compound was a microcrystalline orange powder. The yield was 1.2 gm. (45 per cent), m.p. 152–155° decomposition point (authentic *N*-methyl-3-acetyl-pyridinium reineckate melts at 144–146°, mixed m.p. with the product is 141–143°). The compound analyzed as a dihydrate as follows:

$C_{18}H_{21}ON_9S_4Cr \cdot 2H_2O$ (559.7)

Calculated:	C 32.2, H 4.5, N 22.6, Cr (as CrO_3) 17.8
Found:	C 32.1, H 4.6, N 21.3, Cr (as CrO_3) 17.5

The reineckate could be suspended in water and treated with a suspension of Dowex 1-chloride to yield a yellow solution of 1-methyl-3-acetyl-4-(1-imidazolyl)-pyridinium chloride. Ultraviolet absorption spectra showed in 0.2 N HCl or neutral solutions a maximum at 265 mμ and a shoulder at 275 mμ. In 0.4 N KOH a maximum at 270 mμ and a second maximum at 332 mμ were observed. The minimum was at 295 mμ.

Preparation of p-(1-Imidazolyl)-DPN (Imidazole-DPN)—The reaction mixture, as described in Fig. 4, could be placed on a column of Dowex 1-formate form. For 600 mg. of DPN a column was used with dimensions of 3 × 10 cm. The column was washed thoroughly with water until all the imidazole was eluted. Ferri- and ferrocyanide were held tenaciously to the resin. Gradient elution with formic acid gave a sharp peak (as was determined by absorption at 260 mμ of the eluates) of imidazole-DPN at approximately 0.07 M formic acid. The appropriate fractions were combined and lyophilized to yield 200 mg. of an extremely fluffy powder. Paper chromatography indicated that the material was essentially homogeneous in two solvents tested: (*a*) ethanol and 0.1 N acetic acid (1:1); (*b*) isobutyric acid, concentrated NH_4OH, and water (66:1:33). The R_F value was identical to that of DPN.

Spectral Properties of Imidazole-DPN—The ultraviolet spectra of imidazole-DPN are shown in Fig. 5. It is of interest that the substance still interacts with cyanide; the maximum is, however, shifted from 325 mμ for DPN to 335 mμ for imidazole-DPN. A sample of imidazole-DPN was converted by nitrous acid treatment (14, 40, 41) to deaminoimidazole-DPN. The shift in absorption from 260 to 255 mμ in cyanide clearly indicates that the adenine moiety is converted to hypoxanthine (Fig. 5).

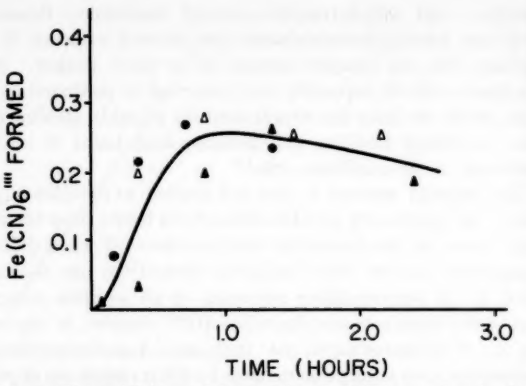

FIG. 4. The oxidation of the imidazole-DPN complex by ferricyanide. 600 mg. (0.6 mmoles by assay) of DPN were dissolved in 6.0 ml. of 7 M imidazole (42 mmoles). 1.8 ml. of a 12 per cent potassium ferricyanide solution were added (0.66 mmoles) together with 0.6 ml. of 2 N KOH (1.2 mmoles). The solution was cooled with ice during the reaction and stirred vigorously. At intervals 0.01 ml. of the mixture was withdrawn and added to 6.0 ml. of a solution containing 1.5 gm. of ferric ammonium sulfate and 1 gm. of sodium laurylsulfonate per l. (39). The density of the prussian blue color formed was estimated after 15 minutes in an Evelyn colorimeter (Rubicon Company), with the use of a No. 660 filter. The different points represent different experiments. A density of approximately 0.25 represents stoichiometric oxidation of DPN.

Chemical Properties of Imidazole-DPN—The nucleotide shows an approximate ratio of 1:2:2:1 of nicotinamide to ribose to phosphate to adenine. The same correct analysis was shown by deaminoimidazole-DPN (Table II). The imidazole-DPN did not show a positive diazo reaction. *N*-methylated imidazole derivatives, however, are known to give negative diazo reactions (42). This point was checked, and it was shown that with conditions under which histidine and carnosine gave the expecte

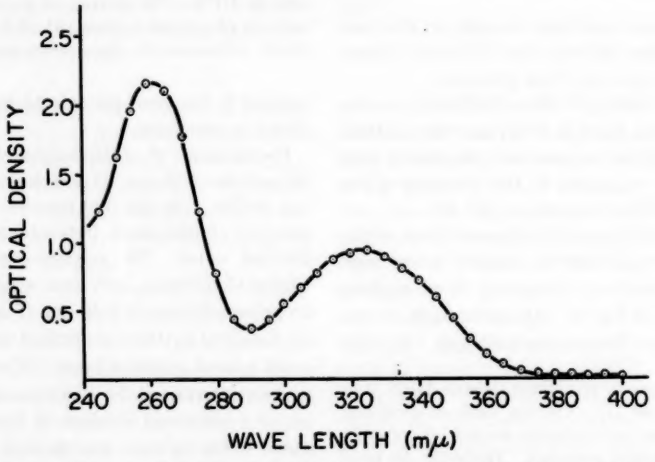

FIG. 3. The absorption spectrum of the acetylpyridine-DPN-imidazole complex with 200 μg. of acetylpyridine-DPN in 2.7 M imidazole. The final volume is 3.0 ml.

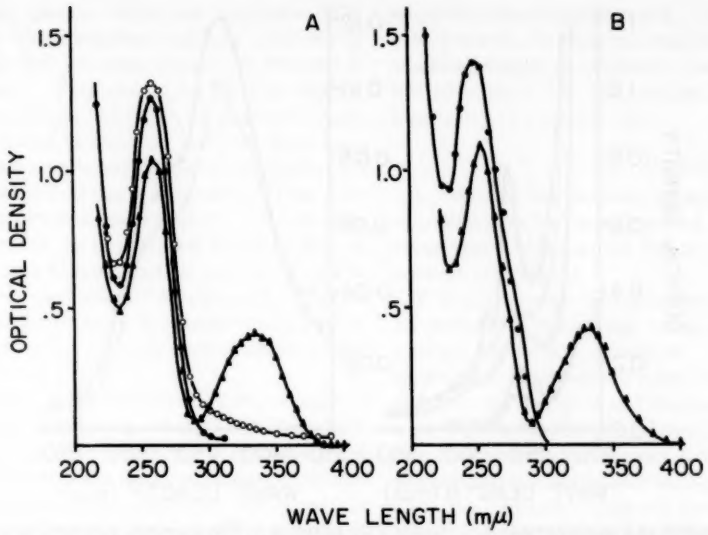

FIG. 5. The absorption spectrum of the oxidized imidazole-DPN complex. *A*, imidazole-DPN, ●——●, 0.1 N HCl, ○——○, and 0.1 N KOH, ▲——▲, in 1 M KCN. *B*, deaminoimidazole-DPN, ●——●, and 0.1 N HCl, ▲——▲, in 1 M KCN. The nucleotide concentration was 130 μg. in 3.0 ml.

color, 1-methyl histidine and anserine did not. It is of interest that 2-methyl imidazole does give a diazo reaction under these conditions.

Upon treatment with hot alkali, imidazole-DPN gives a strongly fluorescent product similar to DPN, with a maximal absorption at 370 mμ. Autoclaving the nucleotide at pH 7.0 yields a substance with growth-promoting activity for *L. arabinosus* in a 60 to 95 per cent yield.

The titration curve of the nucleotide shows only one dissociation between pH 2.0 and 8.5. This dissociation has a pK at 3.7 and presumably reflects the adenine amino group.

Enzymatic Properties of Imidazole-DPN—Imidazole-DPN fails to replace DPN for crystalline yeast alcohol dehydrogenase, crystalline muscle lactic dehydrogenase, or crystalline muscle α-glycerolphosphate dehydrogenase. Also, in concentrations up to 50 times that of DPN, no inhibitory action can be observed.

Neurospora DPNase also fails to attack the new nucleotide. However, this enzyme is 20 per cent inhibited by equimolar concentrations of DPN and imidazole-DPN. Pig brain DPNase and rat liver DPNase, on the other hand, both attacked the imidazole-DPN. The rates of breakdown were respectively 40 and 10 per cent of that of DPN. Nicotinamide at concentrations up to 0.2 M promoted little or no DPN resynthesis from imidazole-DPN by the exchange reaction which these enzymes can catalyze (43, 44). This is analogous to the behavior of acetylpyridine-DPN, which is a good substrate for pig brain DPNase, but one which cannot be converted back to DPN through a nicotinamide-acetylpyridine exchange (13).

Products of Enzymatic Breakdown of Imidazole-DPN—When the reaction mixture of imidazole-DPN and pig brain DPNase was chromatographed on filter paper (Whatman No. 4) with the use of the solvent of ethanol and 0.1 N acetic acid (1:1), two new spots appeared as a result of the action of the enzyme. The incubation mixture of DPN and the enzyme was used for comparison. In this solvent system DPN and imidazole-DPN have identical R_F values at 0.43. The first spot, which was a result of the breakdown of imidazole-DPN, had an R_F of 0.53 and corresponded to adenosine diphosphate ribose. The spot was eluted from the paper and found to be devoid of cyanide-reactive material. It contained adenine, ribose, and phosphate in an approximate ratio of 1:2:2.

TABLE II

Chemical analysis of oxidized imidazole-DPN complex

The adenine was determined by the optical density at 260 mμ in 1 M cyanide, with an extinction coefficient of 1.5×10^4 per mole per l. Nicotinamide was determined by the cyanide adsorption reaction at 335 mμ, with the use of 6.8×10^3 per mole per l. as the extinction coefficient. Residual DPN was estimated by alcohol dehydrogenase assay. The figures are taken in relation to organic phosphate as standard. There was a 4.85 per cent loss of weight upon drying in the imidazole-DPN analysis.

	Imidazole-DPN	Deaminoimidazole-DPN
	μmoles	*μmoles*
Nicotinamide		
Cyanide	0.97	0.80
Microbiological	0.90	
Adenine	0.91	
Ribose	1.50	1.70
Phosphate		
Inorganic	0.13	0
Organic*	2.00	2.00
Total	2.13	2.00
DPN	0	0
Ratio of phosphate to ribose to nicotinamide	1:0.75:0.48	1:0.85:0.40

* Absolute analysis, corrected for inorganic phosphate, yielded the following results for imidazole-DPN:

$C_{24}H_{29}O_{12}N_9O_2 \cdot 2H_2O$ (731.5)

Calculated: P 8.3, H_2O 4.80

Found: P 8.3, H_2O 4.85

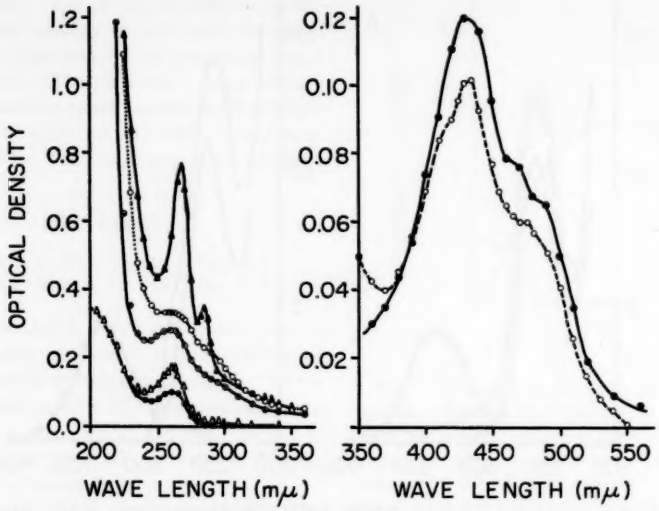

FIG. 6. The spectral properties of 3-carboxamide-4-(1-imidazolyl)-pyridine. The base was prepared as follows: 3 mg. of imidazole-DPN were incubated with 60 mmoles of phosphate buffer adjusted to pH 7.0, together with 8 units of pig brain DPNase, in a fina volume of 1.5 ml. The reaction was followed by withdrawing at intervals 0.1 ml. of the mixture and adding this to 3.0 ml. of 1 M potassium cyanide. The decrease in extinction at 335 mμ was an indication of the hydrolysis. After 1 hour, 60 per cent was hydrolyzed. The tube was placed in a boiling water bath for 3 minutes, the precipitated proteins were removed by centrifugation, and the supernatant was passed over a Dowex 1-chloride column (3 × 1 cm.) after adjusting the pH to 7.8 with disodium hydrogen phosphate. The percolate and 10 ml. of distilled water, used to wash the column, were combined and evaporated to dryness. The residue was taken up in a suitable volume. *Left*, the ultraviolet spectrum of the base. 0.2 N HCl, ⊙——⊙, 0.2 N KOH, ○····○, 1 M KCN, ▲——▲ For comparison the spectrum of nicotinamide at half concentration is shown: 0.2 N HCl, △——△, 1 M KCN or 0.2 N KOH, ●——●. *Right*, spectrum of the cyanogen bromide reaction mixture. The curve with solid circles, for comparison, represents the absorption spectrum of a mixture containing nicotinamide.

The second new spot arising from imidazole-DPN had an R_F of 0.80. Nicotinamide in the control incubation mixture had and R_f of 0.73. This compound, presumably 3-carboxamide-4-(1-imidazolyl)-pyridine, had the spectral characteristics shown in Fig. 6. Larger quantities of the base could be obtained by passing the incubation mixture through a column of Dowex 1-chloride, as described in the figure. The new base was shown to be a tertiary pyridine base by the reaction with cyanogen bromide.

It is of special interest that the cyanogen bromide produced a chromophore which is not grossly different from the one resulting from nicotinamide, although the ultraviolet spectrum is distinctly different. This suggests addition to the pyridine ring rather than alteration of the ring. It is of interest in this respect that the absorption maximum of 1-methyl histidine in alkali at 222 mμ is shifted in cyanide to 230 mμ. This shows an interaction between cyanide ion and substituted imidazole rings.

DISCUSSION

The addition of imidazole derivatives to DPN and other quaternary pyridine bases shows a possible mode of interaction between DPN and proteins. However, although it is not possible to draw definitive conclusions from the observed spectral characteristics of the DPN-imidazole complex, it does not seem likely that the interaction between triose phosphate dehydrogenase and DPN is the result of an imidazole-DPN complex. The mercaptan-DPN complex, which has a maximum of approximately 335 mμ, bears a closer resemblance to the protein-DPN complex at 355 to 360 mμ. This is also suggested by the fact that the protein spectrum is abolished with iodoacetic acid.

The strong interaction of the quaternary pyridinealdoximes with imidazoles is of special interest. Derivatives of this nature are used as reactivators of organophosphate-inhibited proteinases and esterases (45–47). Recent speculations as to the mechanism of hydrolysis by chymotrypsin (48) implicate the proximity of a serine hydroxyl and an imidazole nucleus as the active center of the enzyme. Favorable binding of the quaternary pyridinealdoximes by the imidazole in the active center may therefore conceivably contribute to their reactivating power.

The structure of the reaction product of ferricyanide oxidation of the DPN-imidazole complex as *p*-(1-imidazolyl)-DPN is based upon the following evidence. The new nucleotide has the adenosine diphosphate ribose moiety still intact, which is shown by the unchanged pK of the adenine-amino group, the conversion of the adenine spectrum into a hypoxanthine spectrum by nitrous acid, and the actual isolation of adenosine diphosphate ribose from the action of pig brain DPNase on the molecule. That the nicotinamide ring has not been oxidized to a pyridine is shown by the recovery of nicotinic acid with growth-promoting activity for *L. arabinosus*, the absence of pyridone spectral characteristics, and the positive cyanogen bromide reaction of the pyridine base. The substitution of the ring in the para position is based on analogy to other addition reactions and to the high rate of hydrolysis of the analogue by pig brain DPNase; 2- or 6-substituted derivatives would be expected to give a much slower rate of hydrolysis. Also, substitution at the 2 or 6 position would be expected to give entirely different chromophores after the opening of the ring by cyanogen bromide.

The substitution on the 1 position of the imidazole ring is in-

dicated by the following points. First, the imidazole-DPN fails to show a reaction with diazotized sulfanilic acid; this is similar to the case of 1-methyl imidazole derivatives, whereas 2-methyl derivatives do react. Furthermore, an addition reaction between DPN and imidazole derivatives can be seen with a variety of 2-, 4-, or 5-substituted imidazoles: histidine, histamine, 2-methyl imidazole, benzimidazole, and 2-methyl benzimidazole. On the other hand, 1-methyl histidine fails to react. Thus a reaction at the 1 (3) nitrogen atom seems indicated. This nitrogen has to be a secondary amine, as is indicated by the failure of 2-imidazolone to show an addition reaction with DPN. The absence of a pK_a value for imidazole-DPN below pH 8.5 is also an indication of a substituted nitrogen, since substituted imidazoles in general show higher values of pK for the ring nitrogen than those shown by free imidazole (49).

The actual introduction of an imidazole ring in the molecule is indicated by the enzymatic modification of the DPN derivative, the spectra of the isolated pyridine base from the nucleotide, and the actual elementary analysis of the model substance, 1-methyl-3-acetyl-4-(1-imidazolyl)-pyridinium reineckate. Of course, as before, the para substitution of this latter compound is by analogy rather than by actual determination.

The capacity of imidazole-DPN to add cyanide is a unique example of a double addition to a pyridine ring. It must be noted here, however, that this spectrum slowly fades, so that the double addition product is apparently unstable. There are some spectral indications that the oxidized DPN-dihydroxyacetone analogue can add alkali (37, 38).

SUMMARY

1. The reaction between quaternary pyridinium derivatives and imidazoles has been described. Complexes are formed with absorption maxima in the region previously reported for other addition complexes.

2. The imidazole-diphosphopyridine nucleotide complex could be oxidized by ferricyanide to an oxidized imidazole-diphosphopyridine nucleotide derivative. The properties of this new analogue of diphosphopyridine nucleotide and the probable structure are described and discussed.

3. The imidazole-diphosphopyridine nucleotide addition reaction is discussed with reference to the interaction between diphosphopyridine nucleotide and triosephosphate dehydrogenase.

4. The interaction observed between imidazole and *N*-methyl-2-carboxime-pyridinium iodide is discussed with reference to the ability of this pyridine base to reactiviate enzymes inhibited by organophosphorus compounds.

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Biogenesis of Citrinin*

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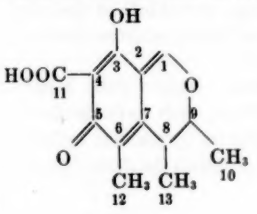
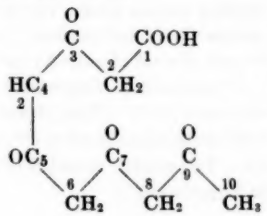
(Received for publication, May 28, 1958)

The occurrence of a transfer of the methyl group of methionine to carbon atom 28 of the sterol skeleton was demonstrated in the case of ergosterol biosynthesis in yeast (1, 2). It seemed advisable to investigate another example of this new biological reaction, and the mold metabolite citrinin (Formula I), $C_{13}H_{14}O_5$, first described by Hetherington and Raistrick (3), was chosen for this investigation. Following the suggestions of Woodward (4) regarding the derivation of certain natural substances from polyacetic acids, the assumption was made that the skeleton of the citrinin molecule consists of a chain of 5 acetic acid molecules combined head-to-tail as presented in Formula II,



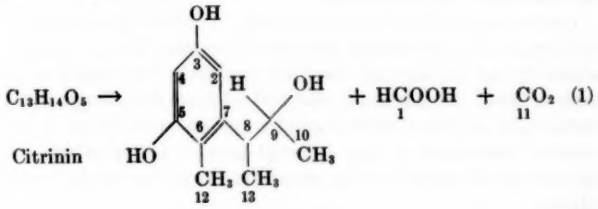
FORMULA I



FORMULA II

and that carbon atoms 1 to 10 are derived from acetic acid, with carbon atoms 1, 3, 5, 7, and 9 coming from carboxyl groups. The remaining 3 carbon atoms (11, 12, and 13) must be derived from some other precursor.

Since degradation of citrinin by acid (3) or alkali (5) treatment follows Equation 1,



Phenol A

the isolation of the 2 crucial carbon atoms, 1 and 11, can easily be accomplished. Carbon atom 1, presumably derived from the carboxyl group of acetate, should be radioactive if sodium acetate-1-C^{14} is used as a precursor, whereas carbon atom 11 should be devoid of radioactivity. Conversely, growing citrinin on methionine-methyl-C^{14} or other 1-carbon sources should produce activity only in carbon atom 11 and none in carbon atom 1.

* This work was supported by United States Public Health Service Grant C321, The Jane Coffin Childs Memorial Fund for Medical Research, an Institutional Grant from the American Cancer Society, and a grant from the Schering Corporation, Bloomfield, New Jersey.

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MATERIALS AND METHODS

Most of the nonradioactive citrinin used for preliminary work and as carrier was provided through the courtesy of Drs. E. B. Hershberg and W. Charney of the Schering Corporation, Bloomfield, New Jersey. Radioactive material was prepared by incubation of *Penicillium citrinum*. Raper (ATCC 1843), in 2 l. Fernbach flasks containing 250 ml. of Czapek-Dox medium. Addition of yeast hydrolysate aided in production of the metabolite. The mold was allowed to grow for 1 week at room temperature before the radioactive substrate was added and the incubation was continued for 1 or 2 weeks after the addition. Citrinin was isolated by acidification of the filtered medium and extraction with chloroform. The crude material was purified by recrystallization from absolute ethanol and from benzene-cyclohexane mixtures. Citrinin was obtained as lemon-yellow needles showing a m.p. of 172°. The yields changed from batch to batch, probably because of the fact that the cultures had to be grown in the laboratory; however, they eventually reached 150 mg. of citrinin per Fernbach flask.

For the experiments presented in Table I the acid degradation of citrinin (3) was carried out on a small scale. Samples of 3 to 6 mg. of citrinin were boiled with 3 ml. of 2 N sulfuric acid for 2 hours, while a slow stream of nitrogen was passed through the system. The liberated carbon dioxide was trapped in a solution of $Ba(OH)_2$. The acid mother liquor was then treated with 200 mg. of HgO and refluxed for another hour to oxidize the formic acid to carbon dioxide, which was collected separately as $BaCO_3$. The degradation for Experiment 3 was performed by boiling 500 mg. of citrinin with 10 ml. of 2 N sulfuric acid for 4 hours and collecting the CO_2 as usual. The acid solution was extracted with ether and then treated with 1 gm. of HgO to oxidize the formic acid. The ether extract yielded 200 mg. of phenol A (m.p., 125–127°) after sublimation and several recrystallizations from chloroform.

The degradation for Experiments 2 and 4 was performed by refluxing the citrinin with aqueous ammonia (5) for 2 hours; the solution was acidified and the subsequent operations were carried out similarly to the acid hydrolysis. The yields of pure phenol A were considerably lower than reported: from 290 mg. of citrinin only 45 mg. of phenol A (m.p., 128–129°) were obtained. The $BaCO_3$ isolated was plated and counted as usual. At least two planchets, containing from 2 to 12 mg. of the carbonate, were made for each substance. The original citrinin and phenol A were counted directly and as $BaCO_3$ after combustion.

TABLE I

Efficiency of utilization of various C^{14} donors in biosynthesis of citrinin (Experiment 1)*

Source of C^{14}	Citrinin	$C^{14}O_2$ recovered	
		Carbon 1	Carbon 11
Sodium bicarbonate	0	0	0
Sodium propionate-2-C^{14}	0.1	0	0
Sodium acetate-1-C^{14}	22.4	3.2	0.1
Serine-3-C^{14}	35.8	0.9	8.1
Sodium formate	55.0	1.4	14.5
Methionine-methyl-C^{14}	100.0	0.6	34.0

* In order to facilitate comparison of the efficiencies of the various substrates, a "molecular activity" was calculated for each substance; this consisted of the observed counts per minute per mmole, as $BaCO_3$, divided by the number of microcuries of substrate used in the experiment. The molecular activity values of all substances were related to the molecular activity of citrinin derived from methionine-methyl-C^{14} by the quotient: molecular activity of substance × 100 divided by molecular activity of citrinin from methionine-methyl-C^{14}.

The fact that acetate provides more carbon atoms than the other substrates was not taken into consideration in these calculations.

TABLE II

Degradation of citrinin-C^{14}

Experiment No.	Source of C^{14}	Substance	Labeled carbon atoms*	Experiment	Theoretical*
				c.p.m./mmole/μc.	
2	Acetate-1-C^{14}	Citrinin	1, 3, 5, 7, 9	120	
		Phenol A	3, 5, 7, 9	95	96
		HCOOH	1	23	24
		CO_2		0	0
3	Methionine-methyl-C^{14}	Citrinin	11, 12, 13	12,700	
		Phenol A	12, 13	10,100	8,470
		HCOOH		0	0
		CO_2	11	5,080	4,230
4	Methionine-methyl-C^{14}	Citrinin	11, 12, 13	26,000	
		HCOOH		170	0
		CO_2	11	8,860	8,670

* Theoretical calculations are based on the assumption of 5 C^{14} atoms in citrinin, 4 C^{14} atoms in phenol A, and 1 C^{14} atom in HCOOH, from acetate-1-C^{14}; and on 3 C^{14} atoms in citrinin, 2 C^{14} atoms in phenol A, and 1 C^{14} atom in CO_2, from methionine-methyl-C^{14}.

RESULTS

Experiment 1. Comparison of Various C^{14} Donors as Citrinin Precursors—Different radioactive carbon sources were added to 250 ml. batches of *P. citrinum* broth and the isolated citrinin was degraded with acid to obtain separate values for carbon atoms 1 and 11. Table I represents a summary of these results. $NaHC^{14}O_3$ and sodium propionate-2-C^{14} gave only insignificant incorporation of radioactivity into citrinin. Whereas, as expected, acetate-1-C^{14} showed incorporation into carbon atom 1 and none into carbon atom 11, radioactivity from formate-C^{14}, serine-3-C^{14}, and especially methionine-methyl-C^{14} appeared in carbon atom 11. Carbon atom 1 is obviously derived from the carboxyl group of acetate, since no other C^{14} source contributes significantly to its radioactivity. Assuming that the nucleus of citrinin is constructed of 5 acetate molecules, the molar radioactivity of C-1 should equal 20 per cent of the molar radioactivity of citrinin. As seen in Table I it equals about 14 per cent. (Compare, however, Experiment 2 (Table II), in which this figure is 19 per cent, which is in excellent agreement with the theoretical value.) Carbon atom 11 contains 34, 26, and 25 per cent, respectively, of the total radioactivity of the citrinin molecule when methionine-methyl-C^{14}, sodium formate-C^{14}, or serine-3-C^{14} are used as substrates. This suggests that the 3 carbon atoms, 11, 12, and 13, come from the same precursor. These results were confirmed in Experiments 2 to 4.

Experiment 2. Degradation of Citrinin Derived from Acetate-1-C^{14}—Three flasks with 250 ml. each were incubated with acetate-1-C^{14} and worked up as before. Phenol A, containing carbon atoms 3, 5, 7, and 9, shows approximately 80 per cent of the total radioactivity of the citrinin and carbon atom 1 shows 20 per cent, whereas carbon atom 11 is devoid of radioactivity, in agreement with theoretical expectation (Table II).

Experiment 3. Degradation of Citrinin Derived from Methionine-methyl-C^{14}—Eight flasks with 250 ml. each were incubated with methionine-methyl-C^{14} and worked up by acid hydrolysis. Results are presented in Table II. This time phenol A (containing carbon atoms 12 and 13) shows two-thirds of the total radioactivity and carbon 11 shows one-third, whereas carbon 1 is now devoid of activity, as expected.

Experiment 4. Degradation of Citrinin Derived from Methionine-methyl-C^{14}—Three flasks with 250 ml. each were incubated with methionine-methyl-C^{14} and worked up by alkaline hydrolysis. The results, recorded in Table II, confirm the data obtained in Experiment 3.

DISCUSSION

The results reported here are in agreement with the assumption that carbon atoms 1, 3, 5, 7, and 9 are derived from the carboxyl group of acetic acid. Carbon atoms 11, 12, and 13 apparently originate with 1-carbon donors, methionine being the most efficient among the compounds tested. Serine and formate may also be used as sources of these atoms, but propionate, acetate, or bicarbonate are not utilized.

Carbon atom 11 is of special interest. It seems probable that it is attached to the citrinin nucleus at the same time as carbon atoms 12 and 13, since all 3 exhibit essentially the same radioactivity level. If this is the case and all 3 are transferred from methionine as intact methyl groups, carbon atom 11 must be oxidized afterwards to the carboxyl group. Intermediates in this process therefore may be present in the culture media from citrinin.

It is interesting to note that in the case of ergosterol as well as in the biogenesis of citrinin described here the methyl group of methionine is attached to a methyl group of one of the acetic acid molecules from which the basic skeletons of these natural substances are derived. Birch *et al.* (6, 7) have also made the same observation in the biogenesis of mycophenolic acid. While this work was in progress, Professor Birch notified the senior author of work done in his laboratory on the biogenesis of citrinin, which led to similar conclusions.

SUMMARY

The biogenesis of citrinin, a product of *Penicillium citrinum*, has been investigated. The evidence suggests that the carbon

skeleton of citrinin is derived from 5 molecules of acetate combined in a head-to-tail arrangement. 2 methyl carbon atoms and the carboxyl carbon atom are derived from 1-carbon donors, of which methionine is the most efficient.

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The Enzymatic Synthesis of δ-Aminolevulinic Acid*

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Received for publication, July 3, 1958

δ-Aminolevulinic acid has been demonstrated to be an intermediate in the synthesis of the porphyrins (1, 2) and related compounds (3). This aminoketonic acid has been postulated to arise biologically from the condensation of "active" succinate (4) with glycine (Reaction 1).

$$\text{"HOOC—CH}_2\text{—CH}_2\text{—COOH"} + \text{CH}_2(\text{NH}_2)\text{COOH} \rightarrow \text{HOOC—CH}_2\text{—CH}_2\text{—CO—CH}_2\text{—NH}_2 + \text{CO}_2 \quad (1)$$

The details of this condensation and the mechanisms involved were not elucidated until very recently. In the last few months, several laboratories have independently reported their preliminary findings demonstrating the enzymatic synthesis of δ-aminolevulinic acid. We have found that particle-free extracts of *Rhodopseudomonas spheroides* and *Rhodospirillum rubrum* cells grown anaerobically in the light catalyze the formation of δ-aminolevulinic acid (5, 6). Sawyer and Smith (7) used extracts of anaerobically grown *R. spheroides*, whereas Gibson (8) demonstrated the synthesis in extracts of aerobically grown *R. spheroides*. Laver and Neuberger (9) and Gibson *et al.* (10) also have reported similar findings with a particulate fraction obtained from erythrocytes of phenylhydrazine-treated chickens.

This communication is a more detailed report on our finding that particle-free extracts of two photosynthetic bacteria, *R. spheroides* and *R. rubrum*, are capable of catalyzing the net synthesis of δ-aminolevulinic acid from succinate and glycine on addition of cofactors (CoA, ATP, pyridoxal phosphate, and Mg^{++} ion) or from succinyl-CoA and glycine on addition of pyridoxal phosphate.

Lascelles (11) has demonstrated that not only are porphyrins synthesized by *R. spheroides* by the sequence of reactions previously elucidated in other cells (12), but also that porphyrins and porphobilinogen accumulate in the medium under particular growth conditions. We were encouraged to investigate this bacterium and related organisms for the enzymes responsible for the synthesis of δ-aminolevulinic acid, for we found that this acid was also present in the medium after the *R. spheroides* cells were suspended in Medium I of Lascelles.

R. spheroides and *R. rubrum* were grown on Medium S of Lascelles (11) under anaerobic conditions and in the light. Extracts of the harvested cells were obtained after the rupture of the bacteria. It can be seen in Tables I and II that extracts of both *R. spheroides* and *R. rubrum* synthesize δ-aminolevulinic acid from succinate and glycine and that there is a marked dependency on pyridoxal phosphate, CoA, ATP, and Mg^{++} ion, especially in dialyzed extracts. Schulman and Richert (13) have shown that pyridoxal phosphate increased heme formation from glycine and succinate in avian erythrocyte preparations. It should also be noted that whereas extracts of *R. spheroides* which contain small particles (centrifuged for 30 minutes) synthesize a fair amount of porphyrins from the formed δ-aminolevulinic acid, little synthesis of porphyrins occurs in particle-free extracts. However, all of the enzymes concerned with the conversion of δ-aminolevulinic acid to porphyrins can be obtained in the soluble fraction, for on the addition of δ-aminolevulinic acid (2.5 μmoles per ml.) to the particle-free extract, porphyrins are formed. This discrepancy can be explained by our finding that the δ-aminolevulinic acid dehydrase in particle-free extracts is markedly inactivated unless glutathione is added to the incubation mixture, while such inactivation does not occur with the particulate fraction.[1] The crude undialyzed extracts of *R. rubrum* are not dependent on CoA as are those from *R. spheroides*. However, as can be seen in Table III, the synthesis of δ-aminolevulinic acid, in extracts of *R. rubrum* which were treated with Dowex 1 (14), was dependent on the addition of CoA.

The δ-aminolevulinic acid was determined by the method of Shuster (15) and was also identified by paper chromatography. The R_F was identical to that found for an authentic sample. In order to document further the identification of δ-aminolevulinic acid, an extract of *R. spheroides* was incubated with all the necessary components and either glycine-2-C^{14} or succinate-1-C^{14}. The formed aminoketonic acid was isolated, after the addition of nonradioactive δ-aminolevulinic acid, as the 2-methyl-3-carbethoxy-4(3-propionic acid) pyrrole derivative (16). It can be seen from Table IV that the derivative of δ-aminolevulinic acid isolated from incubation mixtures to which carrier δ-aminolevulinic acid was added after the incubation period was highly radioactive, and that the radioactivity of the isolated derivative had the same value whether δ-aminolevulinic acid was formed from labeled succinate and unlabeled glycine or from labeled glycine and unlabeled succinate. The equality of radioactivity of these samples demonstrates that 1 mole of succinate condenses with 1 mole of glycine (Reaction 1). The radioactivity found agreed with the value calculated from the determined amount synthesized and the amount of carrier δ-aminolevulinic acid added. It may also be seen from

* This work was aided by grants from the National Institutes of Health, United States Public Health Service (A-1101), from the American Cancer Society, from the National Science Foundation, and from the Rockefeller Foundation.

† On leave from the Nippon Medical School, Tokyo, Japan.

‡ Rockefeller Foundation Fellow, 1957 to 1958.

[1] K. F. Baker-Cohen, Goro Kikuchi, and David Shemin, unpublished observations.

TABLE I

Enzymatic synthesis of δ-aminolevulinic acid in Rhodopseudomonas spheroides and Rhodospirillum rubrum extracts (centrifuged for 30 minutes at 105,000 × g)

The complete system contained 50 μmoles of succinate, 50 μmoles of glycine, 1 μmole of CoA, 0.25 μmole of pyridoxal phosphate, 5 μmoles of ATP, 5 μmoles of $MgCl_2$, 1 ml. of 0.1 M phosphate buffer, pH 6.9, 7.2 mg. of protein of *R. spheroides*, or 12 mg. of protein of *R. rubrum*. The total volume was 2.5 ml.

Incubation mixture	μmoles of δ-ALAC* acid formed/mg. of protein/60 min.		
	Free δ-ALAC	δ-ALAC as porphyrin	Total δ-ALAC
R. spheroides			
Complete system	0.070	0.057	0.127
Minus pyridoxal phosphate	0.020	0.007	0.027
Minus CoA	0.006	0.000	0.006
Minus ATP	0.031	0.022	0.053
Minus ATP, plus ADP	0.040	0.052	0.092
Minus $MgCl_2$	0.085	0.067	0.152
R. rubrum			
Complete system	0.054	0.007	0.061
Minus CoA	0.056	0.006	0.062

* δ-ALAC, δ-aminolevulinic acid.

TABLE II

*Synthesis of δ-aminolevulinic acid**

The incubation mixtures were similar to those in Table I, except that 5.6 mg. of *Rhodopseudomonas spheroides* protein and 9 mg. of *Rhodospirillum rubrum* protein were used.

Incubation mixture	δ-ALAC† formed/mg. of protein/60 min.					
	Free δ-ALAC		δ-ALAC as porphyrin		Total δ-ALAC	
	R. spheroides	*R. rubrum*	*R. spheroides*	*R. rubrum*	*R. spheroides*	*R. rubrum*
	μmoles	μmoles	μmoles	μmoles	μmoles	μmoles
Complete system	0.140	0.023	0.009	0.001	0.149	0.024
Minus pyridoxal phosphate	0.008	0.001	0.002	0.000	0.010	0.001
Minus CoA	0.026	0.012	0.001	0.001	0.027	0.013
Minus ATP	0.002	0.001	0.000	0.001	0.002	0.002
Minus $MgCl_2$	0.062	0.018	0.016	0.001	0.078	0.019

* In extracts obtained after 3 hours of centrifugation at 105,000 × g and dialyzed for 18 hours.

† δ-ALAC, δ-aminolevulinic acid.

Table IV that the addition of carrier δ-aminolevulinic acid at the beginning of the incubation period inhibited its enzymatic formation.

It can be surmised from the foregoing that the formation of δ-aminolevulinic acid may be divided into two over-all reactions: the activation of succinate, followed by the condensation of the "active" succinate with "active" glycine.

Evidence that the extracts of *R. spheroides* and *R. rubrum* contain activating systems for succinate that require CoA, ATP, and Mg^{++} ions is presented in Table V. In these experiments the formation of a hydroxamic acid was investigated. It can

TABLE III

*Effect of Dowex 1 treatment on coenzyme A requirement for Rhodospirillum rubrum extracts**

Incubation mixture	Free δ-ALAC†/mg. of protein/60 min.	
	Before Dowex 1 treatment	After Dowex 1 treatment
	μmoles	μmoles
Complete system	0.023	0.022
Minus CoA	0.012	0.002

* The reaction conditions were similar to those in Tables I and II. The extract was centrifuged for 3 hours at 105,000 × g and dialyzed overnight; 7.4 mg. of protein.

† δ-ALAC, δ-aminolevulinic acid.

TABLE IV

Synthesis of δ-aminolevulinic acid from radioactive substrates in Rhodopseudomonas spheroides extracts

The incubation mixture was similar to those in previous tables except that the substrates were radioactive (0.15 mc. per mmole); 1 mmole of carrier δ-ALAC* was added.

Radioactive substrate	Radioactivity of δ-ALAC derivative (2-methyl-3-carbethoxy-4-(3-propionic acid) pyrrole)	
	Carrier δ-ALAC added	
	Before incubation	After incubation†
	c.p.m.	c.p.m.
Succinate-1-C^{14}	8	833
Glycine-2-C^{14}	39	873

* δ-ALAC, δ-aminolevulinic acid.

† 1 μmole of δ-ALAC was formed in these flasks.

TABLE V

Hydroxamic acid formation

The complete systems contained 50 μmoles of succinate, 1 μmole of CoA, 5 μmoles of ATP, 5 μmoles of $MgCl_2$, 0.7 ml. of neutralized 2.8 M hydroxylamine, 1.0 ml. of 0.1 M phosphate buffer, pH 6.9, 3.3 mg. of *Rhodopseudomonas spheroides* protein, and 9 mg. of *Rhodospirillum rubrum* protein. Extracts were prepared as in Table II.

Incubation mixture	Hydroxamic acid formed/mg. protein/60 min.	
	R. spheroides	*R. rubrum*
	μmoles	μmoles
Complete system	0.590	0.340
Minus $MgCl_2$	0.130	0.045
Minus CoA	0.090	0.033
Minus ATP	0.118	0.003
Minus CoA and ATP	0.109	0.009
Minus succinate	0.030	0.000

be seen that a hydroxamic acid is formed from succinate on addition of these cofactors. The Mg^{++} ion effect shown in Table II is caused by its requirement for succinate activation.

The "active" succinate appears to be succinyl-CoA. This is concluded from the data obtained for Fig. 1 and Table VI.

In Fig. 1 the rate and the amount of δ-aminolevulinic acid formation is far greater from succinyl-CoA than from the components (succinate and CoA) even on the addition of ATP. In *R. rubrum* extracts (see Table VI), succinyl-CoA can substitute for succinate, CoA, and ATP, whereas little synthesis of the δ-aminolevulinic acid occurs from succinate plus CoA. The formation of succinyl-CoA from succinate is more rapid than its formation from α-ketoglutarate, for the latter substrate yields comparatively little δ-aminolevulinic acid when it is added to the complete system in place of succinate. Furthermore, the addition of α-ketoglutarate inhibits the formation of δ-aminolevulinic acid from succinate (Table VII).

The formation of δ-aminolevulinic acid in a particulate-containing extract from *R. spheroides* is greater in the light than in the dark under aerobic conditions (Table VIII). In this experment, the effect of light presumably is caused by a photophos-

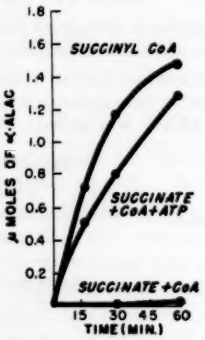

FIG. 1. Rates of synthesis of δ-aminolevulinic acid. Each flask contained *Rhodopseudomonas spheroides* extract (centrifuged at 105,000 × *g* for 3 hours and dialyzed; assayed as 20 mg. of protein) and glycine, 150 μmoles; $MgCl_2$, 15 μmoles, and pyridoxal phosphate, 1.5 μmoles. To the appropriate flasks, as indicated in the figure, 10 μmoles of succinyl-CoA, 10 μmoles of succinate, 10 μmoles of CoA, and 10 μmoles of ATP were also added. The final volume was 12 ml.

TABLE VI

Synthesis of δ-aminolevulinic acid from different components in Rhodospirillum rubrum extracts

The incubation system contained 50 μmoles of glycine, 0.25 μmole of pyridoxal phosphate, 2 μmoles of $MgCl_2$, and 8 mg. of protein. To this incubation mixture the following were added as shown in the first column: 2 μmoles of succinate, 2 μmoles of CoA, 2 μmoles of ATP and 2 μmoles of succinyl-CoA. The total volume was 2 ml. Extracts were prepared as in Table II.

Added components	δ-ALAC* formed after 20 min. of reaction	
	Before Dowex 1 treatment	After Dowex 1 treatment
	μmoles	μmoles
Succinate + CoA + ATP	0.064	0.052
Succinate + ATP	0.022	0.007
Succinate + CoA	0.003	0.001
Succinyl-CoA	0.060	0.052
Succinyl-CoA minus $MgCl_2$	0.066	
Succinyl-CoA minus pyridoxal phosphate	0.000	

* δ-ALAC, δ-aminolevulinic acid.

TABLE VII

Effect of α-ketoglutarate on δ-aminolevulinic acid formation

Incubation mixture	*Rhodopseudomonas spheroides*	*Rhodospirillum rubrum*
	δ-ALAC* (μmoles/mg. of protein)	
Complete system†	0.145	0.033
Minus succinate plus α-ketoglutarate	0.001	0.001
Plus α-ketoglutarate	0.070	0.014
Complete system‡	0.061	0.014
Plus α-ketoglutarate	0.031	0.004

* δ-ALAC, δ-aminolevulinic acid.

† The complete system contained 3.5 mg. of *R. spheroides* protein or 7.2 mg. of *R. rubrum* protein and 50 μmoles of succinate, 50 μmoles of glycine, 1 μmole of CoA, 0.25 μmole of pyridoxal phosphate, 5 μmoles of ATP, 5 μmoles of $MgCl_2$. The final volume was 2.5 ml. 50 μmoles of α-ketoglutarate were added where indicated. The incubation time was 60 minutes.

‡ The complete system contained 2.4 mg. of *R. spheroides* protein or 4.8 mg. of *R. rubrum* protein and 1 μmole of succinyl-CoA, 50 μmoles of glycine, 0.25 μmole of pyridoxal phosphate. The final volume was 2 ml.; 50 μmoles of α-ketoglutarate were added where indicated. The incubation time was 20 minutes. Extracts were prepared as in Table II.

TABLE VIII

Effect of light and anaerobiosis on δ-aminolevulinic acid

The *Rhodopseudomonas spheroides* extract was the supernatant fluid obtained on centrifugation for 30 minutes at 20,000 × *g*. The incubation mixture contained 50 μmoles of succinate, 50 μmoles of glycine, 0.25 μmole of pyridoxal phosphate, 1 μmole of CoA, 5 μmoles of ADP, 5 μmoles of $MgCl_2$, 1 ml. of 0.1 M phosphate buffer, pH 6.9, and 9.8 mg. of protein per flask. The final volume was 2.5 ml.

	Air		Nitrogen	
	Light	Dark	Light	Dark
	μmoles/mg. protein/60 min.			
Free-δ-ALAC*	0.035	0.018	0.010	0.008
δ-ALAC as porphyrin	0.153	0.085	0.039	0.033
Total δ-ALAC	0.188	0.103	0.049	0.041

* δ-ALAC, δ-aminolevulinic acid.

phorylation of added ADP to form ATP which is needed for the activation of succinate. It is worth noting that anaerobiosis under the conditions outlined in Table VIII inhibits δ-aminolevulinic acid formation and that the light effect, though demonstrable, is relatively small.

The cofactor requirement indicates that pyridoxal phosphate is involved in the activation of glycine. It is probable that succinyl-CoA condenses with a stabilized carbanion formed from glycine and pyridoxal phosphate. This stabilized carbanion can be formed either by a loss of a proton or by the loss of CO_2 as indicated in Fig. 2. It would appear from Table IX that the stabilized carbanion is formed by the loss of a proton, for little radioactive CO_2 was formed from glycine-1-C^{14} before the addition of succinate to the incubation flask. Furthermore, it can be seen that the formation of radioactive CO_2 paralleled the

formation of δ-aminolevulinic acid. As yet it cannot be decided from these experiments whether α-amino-β-ketoadipic acid is a free intermediate since decarboxylation may occur by a concerted attack or while the α-amino-β-ketoadipic acid is still in linkage with the pyridoxal phosphate. The inhibition of δ-aminolevulinic acid formation by added δ-aminolevulinic acid would be consistent with the existence of a pyridoxal derivative of δ-aminolevulinic acid.

The extract of *R. spheroides* can be fractionated with ammonium sulfate into two main fractions. The fraction obtained with 30 per cent ammonium sulfate saturation contains mainly the enzyme system concerned with the condensation of succinyl-CoA and the hypothetical pyridoxal phosphate derivative of glycine, whereas the 40 to 65 per cent fraction contains chiefly the succinate-activating system (Table X). The condensing system thus far has been purified 40- to 80-fold, and this purification and that of the activating system will be published in a subsequent paper.

EXPERIMENTAL

Preparation of Enzyme Extract—The photoheterotrophic organisms, *R. spheroides* and *R. rubrum* (obtained from Professor R. Y. Stanier), were grown anaerobically in the presence of light in Medium S of Lascelles in 10-l. bottles. The amount of enzyme per mg. of protein was found to be greater from those organisms grown in a dim light (approximately 50 foot candles) than from those grown in a bright light[2] (see "Discussion"). After the organisms had grown for about 60 to 70 hours (optical density of approximately 0.5 at 680 mμ), they were harvested by centrifugation and washed three times with 0.9 per cent saline. The bacterial cells were broken either by grinding with alumina A-303 (1.5 volumes) or by sonication for 5 minutes of lyophilized preparations. The enzymes can be obtained more readily in a soluble form by the sonication procedure.

The alumina-ground organisms were extracted with 2 to 3 volumes of 0.1 M phosphate buffer, pH 6.9, and the alumina and unbroken cells removed by centrifugation for 10 minutes at 5000 × *g*. This supernatant fluid was then centrifuged at different speeds for varying periods of time. The length of time and the gravitational fields of the centrifugation are indicated in the particular experiments.

The lyophilized organisms were suspended in 20 volumes of 0.1 M phosphate buffer, pH 6.9, and subjected to sonication for 5 minutes in a 9 kc. Raytheon oscillator. The broken cell preparations were centrifuged at 105,000 × *g* for various periods of time.

Incubation Experiments—The extracts were incubated at 34° at pH 6.9 with substrates and cofactors as indicated in the tables and figures. The concentrations of the substrates and cofactors were those which were experimentally determined to yield maximal amounts of the product. The pH used was the optimal pH which was determined experimentally (Fig. 3). After the period of incubation, the reaction was terminated by the addition of one-fifth of the volume of 20 per cent trichloroacetic acid.

Assays—δ-Aminolevulinic acid was determined by the method of Shuster (15), and the porphyrins were determined spectrophotometrically at the wave length of the Soret band ($\epsilon_{\mu M}^{406}$ = 53). The porphyrins were determined in both the trichloro-

[2] T. Abramsky and David Shemin, unpublished observations.

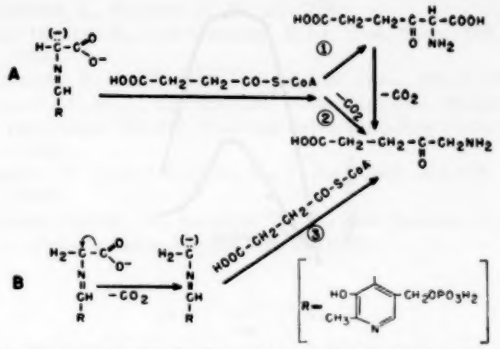

FIG. 2. Suggested mechanisms for δ-aminolevulinic acid formation.

TABLE IX

Decarboxylation of glycine-1-C^{14}

A. The reaction mixture is similar to that of Table I. The extract of *Rhodopseudomonas spheroides* was that obtained on centrifugation for 3 hours at 105,000 × *g* and then dialysis overnight. The amount of protein used was 3.3 mg.; incubation was for 60 minutes.

B. An *R. spheroides* extract (centrifuged at 105,000 × *g* for 30 minutes) containing 40 mg. of protein was incubated with 50 μmoles of succinate, 50 μmoles of glycine-1-C^{14}, 2.5 μmoles of CoA, 0.5 μmole of pyridoxal phosphate, 10 μmoles of ATP, 10 μmoles of $MgCl_2$. The final volume was 4 ml. The radioactivity of the glycine-1-C^{14} was 0.028 mc. per mmole.

A. Effect of succinate

	Radioactivity of CO_2
	c.p.m.
Complete system	203
Minus succinate	5

B. Parallelism of $C^{14}O_2$ and δ-ALAC* formation

Time of incubation	Total δ-ALAC	Radioactivity of CO_2
min.	*μmoles*	*c.p.m.*
40	1.22	555
80	2.08	791
120	2.40	925

* δ-ALAC, δ-aminolevulinic acid.

TABLE X

*Distribution of enzymatic activities obtained in ammonium sulfate fractionation**

Per cent saturated $(NH_4)_2SO_4$	μmoles of products formed/mg. of protein/20 min.	
	δ-ALAC† from succinyl-CoA	Hydroxamic acid from succinate
30% fraction	0.46	0.10
40 to 65% fraction	0.02	0.37
Original extract	0.04	0.33

* Conditions similar to those in previous tables.
† δ-ALAC, δ-aminolevulinic acid.

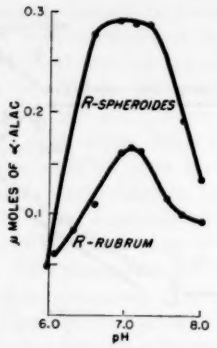

FIG. 3. δ-Aminolevulinic acid synthesis at varying pH values. The *Rhodopseudomonas spheroides* extract (3.3 mg. of protein) was incubated with glycine, 50 μmoles; succinate, 50 μmoles; $MgCl_2$, 5 μmoles; ATP, 5 μmoles; CoA, 1 μmole; pyridoxal phosphate, 0.25 μmole, and phosphate buffer. The final volume was 4.0 ml. The *Rhodospirillum rubrum* extract (9.0 mg. of protein) was incubated with twice the amounts of the additions in the *R. spheroides* extract. The final volume was 4.0 ml.

acetic acid-soluble and -insoluble fractions. Porphyrins were extracted from the trichloroacetic acid precipitate with 5 N HCl. The total amount of δ-aminolevulinic acid formed is the sum of the free acid and that calculated from the amount of porphyrin formed (8 moles of δ-aminolevulinic acid per mole of porphyrin). The formation of hydroxamic acid was determined by the method of Lipmann and Tuttle (17) and the amount of protein determined by the biuret method (18). The C^{14} determinations were carried out as described previously (4).

Preparations—The δ-aminolevulinic acid was converted into the 2-methyl-3-carbethoxy-4-(3-propionic acid) pyrrole derivative by the method of Mauzerall and Granick (16) and succinyl CoA prepared according to the method of Simon and Shemin (19).

Paper Chromatography—An extract of *R. spheroides* was incubated for 90 minutes after the addition of all the required substrates and cofactors. The incubation was terminated by the addition of trichloroacetic acid, and after the ether extraction of the supernatant fluid, it was concentrated to a small volume in a vacuum. A drop of this concentrated solution was placed on Whatman No. 1 paper and chromatographed in an ascending fashion with a phenol-water mixture. The δ-aminolevulinic acid spot was developed by spraying first with ethylacetoacetate and then with 1 N NaOH. After heating the paper for 2 minutes at 100°, it was sprayed with Ehrlich's reagent. The R_F of the spot was the same as that found for an authentic sample which was first mixed with the added components of the incubation mixture. This method of development of the color is an adaptation of Shuster's method for assay of δ-aminolevulinic acid.

DISCUSSION

The findings in this study demonstrate that both substrates, succinate and glycine, are activated by different coenzymes in their condensation to form δ-aminolevulinic acid. The succinate is activated by thiol ester linkage with CoA, whereas the glycine probably is stabilized as a carbanion by pyridoxal phosphate.

The energy requirement for δ-aminolevulinic acid formation would seem to make this step a likely place for the cellular control of prophyrin synthesis. This would be consistent with our observation, mentioned above, that extracts of organisms grown in dim light contained more enzymatic activity than those obtained from organisms grown in a bright light. This finding could be an explanation on an enzymatic level of the observation of Cohen-Bazire *et al.* (20) that organisms grown in dim light synthesized more chlorophyll than those grown in a bright light. Furthermore, we have found that extracts of *R. spheroides* cells grown aerobically in the dark were far less active for the synthesis of δ-aminolevulinic acid than extracts obtained from cells grown anaerobically in the light. In addition, the synthesis of δ-aminolevulinic acid was inhibited when extracts of aerobically dark-grown cells were added to extracts from anaerobically light-grown cells. This inhibition is abolished on heating the extract of the aerobically grown cells.[2] Similar findings were obtained with the photosynthetic sulfur bacteria, Chromatium.[3] Extracts of this organism did not appear to synthesize δ-aminolevulinic acid and inhibited the synthesis in extracts of the anaerobically grown *R. spheroides*. We have obtained further evidence for the presence of an inhibitor during enzyme purification. The total activity for δ-aminolevulinic acid synthesis is greatly enhanced on the first ammonium sulfate fractionation of the crude extract of anaerobically grown cells. The nature of this inhibitory reaction is being investigated. *R. spheroides* organisms appear to overproduce intermediates in porphyrin synthesis under certain conditions, and this phenomenon is biochemically similar to the occurrences in the hereditary disease of acute prophyria. If one is permitted to extrapolate the findings in *R. spheroides* to that of acute porphyria, it might be suggested that patients in the acute phase of this desease may have less of an inhibitor similar to or identical with that in the *R. spheroides*.

SUMMARY

The enzymatic formation of δ-aminolevulinic acid in particle-free extracts of *Rhodopseudomonas spheroides* and *Rhodospirillum rubrum* has been studied. It has been demonstrated that succinate is converted to succinyl-coenzyme A and this activated form of succinate condenses with a pyridoxal phosphate derivative of glycine to form δ-aminolevulinic acid and carbon dioxide.

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The Activation of Adrenal Phosphorylase by the Adrenocorticotropic Hormone*

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(Received for publication, July 21, 1958)

A theory has been proposed to explain, in part, the effects of the adrenocorticotropic hormone on the adrenal cortex (1). This theory is based principally on the fact that phosphorylase activity is rapidly increased in adrenal tissue stimulated by this hormone. The present work was performed to study the mechanism by which ACTH[1] brings about this increase in phosphorylase activity. The experiments described here were designed to investigate the possibility that adrenal phosphorylase stimulation by ACTH is mediated through 3′,5′-AMP, as is the stimulation of hepatic phosphorylase by epinephrine and glucagon (2). It was found that ACTH added to adrenal tissue slices caused an increase in the tissue level of the cyclic nucleotide. When 3′,5′-AMP was added to adrenal tissue slices, the phosphorylase activity of the adrenal tissue was increased. These experiments indicated that 3′,5′-AMP does indeed serve as an intermediate agent in the ACTH-induced stimulation of phosphorylase.

EXPERIMENTAL

Incubation of Slices—Preparation and incubation of beef adrenal cortical tissue slices were as previously described with a ratio of medium volume (in milliliters) to tissue weight (in grams) of approximately 12 (1). A saline phosphate medium at pH 7.4 proved satisfactory for the incubation studies described here. Commercial ACTH was routinely added at a concentration of 0.2 to 0.4 unit per ml. of incubating medium.

Incubation of Particulate Preparation—Tissue slices prepared from beef adrenal cortices were homogenized in a Potter-Elvehjem type homogenizer in a 10 per cent sucrose solution. The preparation was strained through gauze and centrifuged at 1200 × *g* for 10 minutes. The precipitate was washed twice with 10 per cent sucrose and then suspended in 3 volumes of 10 per cent sucrose. To this suspension the following substances were added to the final concentrations indicated: $MgSO_4$, 2.5×10^{-3} M; ATP, 2×10^{-3} M; tris(hydroxymethyl)aminomethane buffer, pH 7.2, 4×10^{-2} M; NaF, 10^{-2} M; and caffeine, 6.6×10^{-3} M. The suspension was incubated with shaking at 30° for 15 minutes.

Ion Exchange Chromatography—Chromatography of 3′,5′-AMP on ion exchange columns was done according to the procedure of Sutherland and Rall (3).

Paper Chromatography—Paper chromatography of 3′,5′-AMP was done with Whatman No. 1 filter paper and the alcohol-ammonium citrate system of Greenberg (4), at pH 4.4.

Assay Techniques—Phosphorylase, phosphoglucomutase, and glucose-6-phosphate dehydrogenase activities were determined by techniques previously described (1).

The levels of 3′,5′-AMP were determined in the following manner. Tissues for assay were placed in water 1 to 5 times the weight of the tissue previously heated to 100°. The tissue and water were then heated at 100° for 10 to 12 minutes. The contents of the vessel were frozen, thawed, homogenized, and centrifuged. The supernatant fluid was assayed directly or concentrated by passing the heated extract over a column of Dowex 2 resin and eluting the 3′,5′-AMP with small volumes of 0.05 M HCl. The 3′,5′-AMP was assayed in the liver phosphorylase system described by Rall and Sutherland (5).

RESULTS

Presence of 3′,5′-AMP in Adrenal Tissue—A particulate preparation from adrenal cortical tissue was incubated in the presence of ATP, $MgSO_4$, NaF, and caffeine, as described above. At the end of the incubation period, a boiled extract of the suspension contained significant amounts of 3′,5′-AMP. Identification of the compound was based on the following criteria. (*a*) The purified extract was biologically active; that is, it stimulated the activation of hepatic phosphorylase in the standard assay system. (*b*) This biological activity was present after heating in water at 100° for 10 minutes, which indicated a heat stability like that of authentic 3′,5′-AMP (3). When the extract was passed over columns of Dowex 2, Dowex 50, and a second Dowex 2 column and was eluted with 0.05 M HCl, the biological activity was found in eluted fractions that were the same as those with authentic 3′,5′-AMP (4). Ultraviolet absorption of active fractions showed a 280 to 260 mμ ratio of 0.24 to 0.29, and these ratios are comparable to those obtained with 3′,5′-AMP and other adenine-containing compounds (5). Mobility of the compound in an alcohol-ammonium citrate paper chromatographic system showed ultraviolet-absorbing material with an R_F of 0.363, as compared with an R_F of 0.351 for 3′,5′-AMP. In the same system 3′-AMP had an R_F of 0.318, and 5′-AMP had an R_F at 0.253. On the basis of these criteria it was concluded that 3′,5′-AMP is present in, or can be synthesized by, adrenal cortical tissue under the conditions of the experiment.

* This investigation was supported in part by funds (Grant No. A-1256) from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service.

[1] The abbreviations used are: ACTH, adrenocorticotropic hormone; 3′,5′-AMP, adenosine-3′,5′-monophosphate; 2′,3′-AMP, adenosine-2′,3′-monophosphate.

Effect of ACTH on Level of 3′,5′-AMP in Adrenal Tissue Slices—Adrenal tissue slices which were incubated for 20 minutes with or without added ACTH were assayed for their content of 3′,5′-AMP. Table I shows the results of one such experiment. It can be seen that in this particular experiment ACTH caused the tissue level of 3′,5′-AMP to be 3 to 5 times higher than in the control slices. It was found that the increase in tissue content of 3′,5′-AMP in the presence of ACTH was the result of a net synthesis of the compound rather than of prevention of the destruction of the compound already present in the tissue. This was demonstrated by determining the tissue content of 3′,5′-AMP at the beginning as well as at the end of incubation. Table II shows the results of such an experiment. It can be seen that ACTH caused a net accumulation of 3′,5′-AMP.

The specificity of ACTH in eliciting this response was studied by adding glucagon, epinephrine, and insulin to slices in the same manner as ACTH was added. It can be seen from Table III that these hormones did not cause accumulation of 3′,5′-AMP to a detectable level. In this experiment it will be noted that the 3′,5′-AMP levels in control slices, as well as in those slices treated with glucagon, insulin, and epinephrine, were too low to be assayed by the technique used. A comparison of Tables II and III reveals the typical variability which was found from experiment to experiment in the tissue levels of 3′,5′-AMP. When low tissue levels were encountered in experiments which involved only small amounts of tissue, there was insufficient material to allow concentration to a point where control levels could give a reading in the assay system. The cause of the variation in 3′,5′-AMP levels is not known.

The biologically active, heat-stable factor that was obtained in increased amounts in slices treated with ACTH, and that was presumably 3′,5′-AMP, behaved on ion exchange columns like 3′,5′-AMP. Also, biological activity of the factor was destroyed by an enzyme prepared from heart which is known to hydrolyze 3′,5′-AMP rapidly (3). Thus, although insufficient quantities were obtained from slices to give a positive identification, there is reasonable certainty that it was, indeed, 3′,5′-AMP which was measured in the bioassay.

Effect of 3′,5′-AMP on Adrenal Phosphorylase—When 3′,5′-AMP was added in a concentration of 8×10^{-4} M to the medium in which adrenal slices were incubated for 20 minutes, the phosphorylase activity of the slices increased just as it did when ACTH was added. Table IV shows the results of one such experiment.

The specificity of this reaction was determined in two ways. First, the levels of activity of two other enzymes, phosphoglucomutase and glucose-6-phosphate dehydrogenase, were assayed

TABLE I

3′,5′-AMP levels in adrenal tissue slices

Values are given as molarity of the compound within the tissue slices.

Control slices	ACTH-treated slices
3.0×10^{-6}	11×10^{-6}
2.0×10^{-6}	11×10^{-6}
2.5×10^{-6}	10×10^{-6}
Mean 2.5×10^{-6}	10.7×10^{-6}

TABLE II

3′,5′-AMP content of tissue slices

Values are given as the molarity of the compound within the tissue slices. Each value was determined in a 5-gm. group of adrenal tissue slices.

Conditions	3′,5′-AMP level
At start of incubation	0.4×10^{-7}
Incubated for 20 minutes without ACTH	0.56×10^{-7}
Incubated for 20 minutes with ACTH	3.56×10^{-7}

TABLE III

Effect of various compounds on 3′,5′-AMP levels in adrenal slices

Values are given as molarity of the compound within the tissue. All values given are the means of duplicate determinations.

Material added	3′,5′-AMP level
KCl	0
ACTH	5×10^{-6}
Epinephrine	0
Insulin	0
Glucagon	0

TABLE IV

Effect of added 3′,5′-AMP on phosphorylase activity of adrenal tissue slices

Phosphorylase values are expressed as micromoles of phosphate released from glucose-1-PO_4 by 1 gm. of tissue in a 30-minute assay. The values given are the means of triplicate determinations.

Material added	Phosphorylase activity
KCl	29
ACTH	72
3′,5′-AMP	66

TABLE V

Specificity of 3′,5′-AMP in activation of phosphorylase in adrenal tissue slices

Phosphorylase values are expressed as in Table III. Values represent means of duplicate determinations.

Material added	Phosphorylase activity
Experiment 1	
KCl	37
ACTH	77
3′,5′-AMP	130
ATP	47
5′-AMP	50
Adenosine	47
Experiment 2	
NaCl	26
ACTH	59
3′,5′-AMP	67
2′,3′-AMP	36

in slices which had been incubated with added 3′,5′-AMP. The phosphorylase activity of these slices was doubled by the addition of the cyclic nucleotide, but the levels of the other two enzymes were not significantly affected. The other study relating to specificity was an attempt to substitute related compounds for the 3′,5′-AMP. Compounds studied were adenosine, ATP, 5′-AMP, and adenosine-2′,3′-monophosphate. Table V shows the results of two such experiments. It can be seen that none of the compounds tested stimulated phosphorylase as 3′,5′-AMP did. Several of the compounds showed possible weak stimulating effects, but these were considered not to be significant.

DISCUSSION

These experiments indicate that ACTH causes the accumulation of 3′,5′-AMP in adrenal cortical tissue. Furthermore they show that the latter compound is capable of causing an increase in phosphorylase activity in the adrenal. It would appear from these findings that the activation of adrenal phosphorylase which results from ACTH stimulation is mediated through 3′,5′-AMP. This is analogous to the action of epinephrine and glucagon, which activate hepatic phosphorylase by means of the same compound. In the instance of the liver it has been shown that epinephrine and glucagon act on a particulate fraction of the cell to cause an accumulation of 3′,5′-AMP. Although it has been demonstrated that a comparable cellular fraction from the adrenal, under certain conditions, is capable of synthesizing 3′,5′-AMP, it has not been established as yet that ACTH acts on this particulate system. However, this would seem to be a good possibility, and investigation of this aspect of ACTH action is continuing.

Although phosphorylase activation in the liver and in the adrenal cortex is brought about by the same nucleotide, there seems to be no cross-over in hormone specificity; that is, ACTH does not activate hepatic phosphorylase, and epinephrine and glucagon do not activate adrenal phosphorylase. Thus it seems that the tissue specificity of these hormones resides in their ability to cause an accumulation of 3′,5′-AMP within a particular organ.

On the basis of these and earlier experiments (1), a general outline of a theory can be proposed to explain, in part, the action of ACTH on the adrenal cortex. Accordingly, ACTH causes an accumulation of 3′,5′-AMP within the adrenal cortex. This compound in turn stimulates the activation of phosphorylase. The higher level of phosphorylase activity permits glycogen to be broken down at an increased rate to glucose-1-phosphate. This ester is rapidly converted to glucose-6-phosphate, which is metabolized primarily by the dehydrogenase system. As a result of this dehydrogenase activity, reduced TPN is generated. Reduced TPN provides the necessary energy for a number of steps involved in corticosteroid synthesis. As these synthetic processes continue and corticosteroids accumulate within the adrenal cells, they begin to pass from these cells into the circulation.

The work by Koritz and Péron which showed that the addition of glucose-6-phosphate and TPN to rat adrenal slices increases the output of corticosteroids tends to confirm one aspect of the above theory, that is, that reduced TPN stimulates corticosteroid biosynthesis (6).

SUMMARY

1. When adrenocorticotropic hormone was added to incubating slices of beef adrenal cortex, adenosine-3′,5′-monophosphate, accumulated within the tissue.

2. Phosphorylase activity was increased when adenosine-3′,5′-monophosphate was added to incubating adrenal cortical tissue slices.

3. From these observations, it was concluded that the activation of adrenal cortical phosphorylase by adrenocorticotropic hormone is mediated through adenosine-3′,5′-monophosphate.

4. The general outline of a theory on the mechanism of action of adrenocorticotropic hormone was presented.

Acknowledgments—The continued interest and help of Dr. Earl W. Sutherland is deeply appreciated. The technical assistance of Miss Charlotte H. Fox is gratefully acknowledged.

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The Kinetics and Inhibition of Cytochrome Components of the Succinic Oxidase System

III. CYTOCHROME *b**

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(Received for publication, March 25, 1958)

In the course of studies by Keilin and Hartree (1) on succinic oxidase preparation of heart muscle particles, it was observed that the kinetics of reduction of cytochrome *b* were not in accord with some rather fundamental criteria that must be met for an electron-transferring component in the respiratory chain (2). On the other hand, studies of the kinetics of cytochrome *b* in intact yeast cells showed its behavior to be in reasonably good agreement with that expected of an electron-transferring component (3). Studies of cytochrome *b* in isolated rat liver and heart mitochondria afforded an explanation for this apparent inconsistency, since in these efficiently phosphorylating particles cytochrome *b* has an active function (4, 5). At some point in the disruption of the heart sarcosomes occasioned by the Keilin and Hartree procedure, the function of cytochrome *b* is altered so that most of the electrons bypass it in their path from substrate to oxygen. Furthermore, at some stages in the disruption, phosphorylative activity is diminished and then is lost completely. To the extent that the kinetic response of cytochrome *b* of the Keilin and Hartree preparation does not represent the behavior of the same component when it is a part of the electron-transfer chain of the phosphorylating particle, its behavior in this preparation is that of an artifact. Nevertheless, many useful kinetic data (2) can be obtained with the above mentioned preparation, which is, incidentally, very convenient for spectroscopic studies because of the degree of light scattering, which is relatively small as compared to that of mitochondria. Such kinetic data now seem to have a real value in our understanding of what has occurred to the reactivity of cytochrome *b* in the preparation of the Keilin-Hartree particles. The results are also useful in evaluating the relationship of cytochrome *b* to the succinic-cytochrome *c* reductase activity of portions of the respiratory chain that have been isolated by various preparative procedures. Such studies may shed light on the mechanism of electron transfer in interactions between flavoproteins and cytochromes, even though the actual reactions involved are not the same as those that occur in the mitochondria during oxidative phosphorylation.

This paper describes methods for the spectroscopic study of cytochrome *b*, the kinetics of its reduction and oxidation by succinate and fumarate, and an unexpected activation of the succinate-cytochrome *b* reaction by antimycin A treatment. Antimycin A treatment also permits the identification of what appears, from its distinctive absorption band, to be a "modified cytochrome *b*."

* This research was supported in part by a grant from the United States Public Health Service.

METHODS

The succinic oxidase preparations were carried out by a modification of the Keilin-Hartree procedure (1), as discussed in a previous communication (6). The $(K_4)_{a_3}$ values[1] for the preparations were in the range of 70. The temperature was 26°.

The experiments reported here cover a period of approximately 5 years, hence the spectroscopic recordings were made with several instruments as they were developed. Difference spectra were obtained with the split beam recorders of Yang and Legallais (7) and Chance (8), but the records actually presented here, such as those in Fig. 1, were obtained with an instrument of higher resolution (spectral interval of about 1.5 mμ). Such split beam instruments automatically plot the spectrum representing the difference in absorbancy between two samples that are identical except for the state of oxidation of their respiratory enzymes. Since we have been recording difference spectra for some time with an arrangement of end-on photocell and a cuvette that minimizes errors attributable to light scattering, the appreciable errors discussed by Keilin and Hartree (9) are not significant and their laborious procedure for cytochrome assay is therefore unnecessary.

The reaction kinetics illustrated by the subsequent figures in the paper were obtained with a double beam apparatus employing two quartz monochromators (8). This apparatus records, as a function of time, the difference between changes of absorption at two predetermined wave lengths.

RESULTS

Spectra—The spectrum representing the difference in absorbancy between the anaerobic, succinate-reduced forms and the oxidized forms of the heart muscle preparation is shown in Fig. 1*A*. The noteworthy features of this difference spectrum are:

1. The preparation represented is a "low cytochrome *c*"

[1] The symbol $(K_4)_{a_3}$ is related to the turnover number of cytochrome a_3: $(K_4)_{a_3}$ = μM O_2/sec./ΔD_{445}, where the respiratory rate is measured by the platinum microelectrode and ΔD_{445} is the absorbancy change at 445 mμ on reduction of cytochrome a_3 (5).

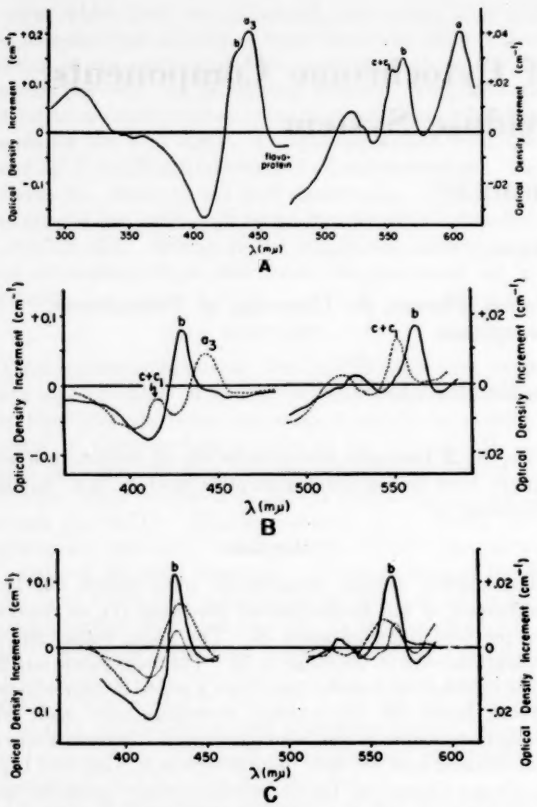


FIG. 1. Spectra representing differences in absorbancy between the reduced and oxidized states of various components of the Keilin and Hartree heart muscle preparation.

A, the difference between the succinate-reduced components and the oxidized material. The absorption maxima are so identified in the figure.

B, illustration of the technique of separating the absorption bands of cytochromes *c*, c_1, *a*, and a_3 from those of cytochrome *b* and flavoprotein. The former set of components was reduced by ascorbate treatment of the cyanide-inhibited material, and the absorption bands of $c + c_1$ and a_3 were obtained. It should be noted that the band of a_3 corresponds to the difference between the oxidized and reduced forms of the cyanide-inhibited material. Absorption bands due to cytochrome *b* and flavoprotein were obtained by succinate treatment of the components already reduced by ascorbate, and the results are represented by the *solid traces*, which show the absorption bands of cytochrome *b* at 562 and 430 mμ.

C, chemical treatments of heart muscle preparations that permit differentiation of absorption bands usually attributed to cytochrome *b*. The *solid trace* represents the absorption bands that appeared upon addition of succinate to the material pretreated with QO and shows the absorption bands attributable to cytochrome *b* (note that the absorption peak of cytochrome *b* lies at 564 mμ, as contrasted with 562 mμ in Fig. 1*B*, *solid trace*). The Soret band also lies at a slightly longer wave length than that of cytochrome *b* (Fig. 1*B*, *solid trace*). *Dotted trace*, a spectrum of the pigment obtained when antimycin A was added to material already reduced by succinate; the absorption peaks are at 566 and 432 mμ. *Dashed trace*, the components that were not reduced by successive treatment with ascorbate, succinate, and antimycin A. Addition of dithionite resulted in a broad absorption band with a peak at 556 to 566 mμ and a very broad Soret band with a peak at 435 mμ.

The experiments were carried out in 0.15 M phosphate buffer, pH 7; the value of $(K_4)_{a_3}$ for the preparation was 55. Cyanide concentration, 3 mM; ascorbate concentration, 1 mg. per ml.; succinate concentration, 8 mM; antimycin A concentration, approximately 10 μM; QO concentration, approximately 20 μM; dithionite concentration, 1 mg. per ml. (Experiment 687).

preparation in which the absorption band of cytochrome c_1 at 554 mμ is clearly recorded (10), whereas that of cytochrome *c* at 550 mμ appears only as a broadened band of cytochrome c_1 in the difference spectrum at room temperature. At low temperatures the spectrum clearly shows the presence of cytochrome *c* in such preparations (11).

2. The spectrum has been carried into the ultraviolet region in order that the content of ultraviolet-absorbing components in the DPN-free succinic oxidase preparation may be evaluated to show the extent to which such absorption interferes with pyridine nucleotide measurement in sarcosomes and mitochondria (5). The absorption maxima are 604, 562, 553, and 444.5 mμ for cytochromes *a*, *b*, c_1 (and *c*), and a_3, respectively.[2]

The sequence of action of the cytochrome components of the nonphosphorylating succinic oxidase preparation previously presented (13) is enlarged to include cytochrome c_1 (14),

$$\text{succinate} \longrightarrow \text{dehydrogenase} \longrightarrow f \longrightarrow c_1 \longrightarrow c \longrightarrow a \longrightarrow a_3 \longrightarrow O_2$$
$$\downarrow$$
$$b$$

The cytochrome *b* component can be studied without interference from the other components by adding, first, a substrate (ascorbate) that chemically reduces the components between c_1 and oxygen (Fig. 1*B*, *dashed trace*) and then a second substrate[3] (succinate) (Fig. 1*B*, *solid trace*) that reduces the components of the chain lying between dehydrogenase and cytochrome c_1. Alternatively, an inhibitor such as antimycin A or QO[4] (15) (Fig. 1*C*, *solid trace*) intercepts electron transfer between dehydrogenase and cytochrome c_1. In both of these cases the addition of succinate reveals absorption bands attributable to the flavoprotein and the cytochrome *b* components (Fig. 1*B* and *C*, *solid traces*). In Fig. 1*B* (*solid trace*) the absorption band is at 562 mμ, whereas in Fig. 1*C* (*solid trace*) it is at 564 mμ. This is a significant discrepancy (see below). Ascorbate treatment of the cyanide-inhibited material produces the absorption bands shown in Fig. 1*B* (*dashed trace*), in which the combined peaks of cytochromes *c* and c_1 appear at 552 mμ. The characteristically small Soret band of cytochrome $c + c_1$ is clearly distinguished at about 417 mμ. Inasmuch as cyanide is present the Soret band of cytochrome *a* is not as prominent as in Fig. 1*A*.

Fig. 1 illustrates the use of three successive chemical treatments to reveal different "*b*" compounds. The results may be compared with the results obtained with treatments used for liver mitochondria (16, 17). In the first step succinate is added to

[2] This spectrum is very similar to that of the "low cytochrome *c*" preparation discussed in Paper II of this series (12), in which the "cytochrome *c*" absorption band was displaced from that of purified cytochrome *c* at 550 mμ to about 553 mμ (Fig. 3*C*, (12)).

[3] Actually two cuvettes are employed. Both are treated with ascorbate, and a base-line is plotted. Succinate is then added to one cuvette and the difference spectrum is plotted.

[4] The abbreviation used is: QO, 2-*n*-heptyl-4-hydroxyquinoline N-oxide.

the ascorbate-reduced sample and the absorption bands of cytochrome *b* at 562 and 430 mμ are clearly distinguished; a band of flavoprotein is also observed at 450 mμ (Fig. 1*B*, *solid trace*). The second chemical treatment (Fig. 1*C*, *dotted trace*) consists of the addition of antimycin A to the sample to which succinate has already been added, revealing a new absorption band at 566 mμ with a distinct shoulder at 560 mμ and a Soret band at 432 mμ (*cf.* (2)). In the third step hydrosulfite is added to the solution already treated successively with ascorbate, succinate, and antimycin A (Fig. 1*C*, *dashed trace*), with the result that a broad absorption band is observed in the visible (12) and Soret regions at about 558 mμ and at 435 mμ, respectively. Examination of this pigment by a difference spectrum at liquid nitrogen temperatures does not show detectable amounts of cytochromes c_1 or *b*. This three-step process makes possible an accurate assay of the pigments of the heart muscle preparation and is simpler than the "optical resolution" method (18, 19). Also, the first three steps are unaffected by hemoglobin contamination of the preparation.

The new pigment absorbing at 566 mμ is not formed specifically by antimycin A; QO is equally effective. Ascorbate is not required for the reaction leading to the band at 566 mμ. The peak at 564 mμ, shown in Fig. 1*B* (*solid trace*), can be accounted for in terms of a mixture of compounds with peaks at 562 and 566 mμ, respectively. The 566 mμ absorption band may also be obtained as a component of the broad band caused by the addition of dithionite to succinate-reduced heart muscle preparation. These experiments identify the 566-mμ pigment as a component of the heart muscle particles that can be reduced directly by dithionite or that becomes reducible by succinate in the presence of antimycin A or QO.

Kinetics and Equilibrium of Cytochrome b Reduction—Titration of cytochrome *b* with succinate in a cyanide-inhibited system is illustrated in Fig. 2*A*. The downward deflection of the trace after successive additions of succinate is attributable to increased absorption of the type indicated by Fig. 1*C* (*solid trace*). Under the conditions of the experiment, only 45 μM succinate is required to give half-maximal reduction of cytochrome *b*. The fact that the addition of dithionite results in a further absorbancy change after the end point of the succinate titration has been reached supports the data presented in Fig. 1*C*, which show the presence of an additional compound reduced in this reaction. The increased absorbancy induced by dithionite cannot be accounted for by an incomplete reduction of cytochrome *b* (562 mμ) by succinate. The finding on dithionite is contrary to the assumption implicit in Ball's oxidation-reduction calculations (20): that cytochrome *b* is not completely reduced, even by a very large succinate to fumarate ratio, unless dithionite is added.

The fumarate oxidation of succinate-reduced cytochrome *b* in the cyanide-inhibited system is illustrated in Fig. 2*B*. As in Fig. 2*A*, measurements were made at 562 mμ with reference to 575 mμ. Cytochrome *b* is initially reduced by addition of 138 μM succinate. Successive additions of fumarate cause a decrease of absorbancy at 562 mμ until a plateau is reached in the vicinity of 25 mM fumarate. Half-maximal oxidation is obtained with 5.8 mM fumarate. A 50 per cent reduction is obtained with a fumarate to succinate concentration ratio of 42; this corresponds to a much higher oxidation-reduction potential than that given by Ball (20) (see "Discussion").

The reactivity of cytochrome *b* towards succinate in the material treated with antimycin A (*cf.* Fig. 1*C*) is indicated by the experiments illustrated in Fig. 3. Since the absorption band is shifted somewhat to longer wave lengths, we have

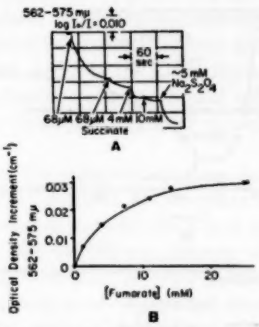


FIG. 2. The kinetics and equilibrium of cytochrome *b* reduction.

A, succinate titration of cyanide-inhibited heart muscle preparation. The double beam spectrophotometer was used to measure the increased absorbancy at 562 mμ with respect to 575 mμ. The additions of succinate in μM concentrations are indicated. At the end of the titration, dithionite was added in order to reduce pigments not reducible by succinate (*cf.* Fig. 1*C*, *dashed trace*).

B, fumarate oxidation of succinate-reduced cytochrome *b* in the cyanide-inhibited system. Conditions were similar to those given in Fig. 2*A*. The initial succinate concentration was 138 μM; cyanide, 0.9 mM (Experiments 286d-3, 286d).

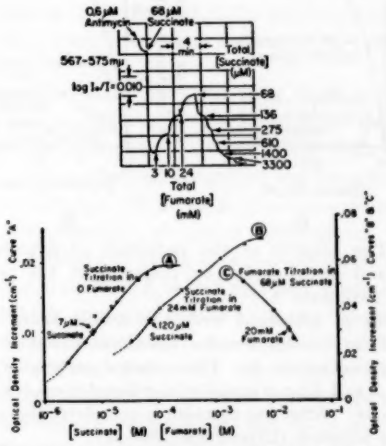


FIG. 3. Titration of cytochrome *b* in the antimycin A-inhibited system, with succinate and with fumarate. *Upper*, a rise of the trace indicates a decrease of absorbancy at 567 mμ as measured with reference to 575 mμ; this corresponds to an oxidation of cytochrome *b*. The various additions of succinate and fumarate are indicated on the diagram. Note that the time scale is one-fourth that shown in Fig. 2*A*. The antimycin A concentration was 10.6 μM (Experiment 279d). *Lower*, effect of fumarate upon the apparent equilibrium of succinate and cytochrome *b* in the antimycin A-inhibited system. The succinate titrations in the presence of fumarate are taken from the upper figure. The succinate titration in the absence of fumarate was made in the presence of 14 μM antimycin A and 0.9 mM cyanide (Experiments 279d and 286f).

measured the absorbancy changes at 567 mμ with reference to 575 mμ. The first addition consists of antimycin A, which causes a small absorbancy change. Subsequent addition of 68 μM succinate causes a large and rapid absorbancy increase. In fact, a more detailed study (plotted in *Curve A*, lower portion) shows that only 7 μM succinate is required for half-maximal reduction as compared with 45 μM in the absence of antimycin A. Back-titration with reasonably low concentrations of fumarate is next carried out, the addition of 24 mM fumarate resulting in nearly a 50 per cent disappearance of the absorption caused by the addition of succinate. Further titration (*Curve B*) with succinate is begun, and an end point is reached with the addition of about 10 mM succinate. The concentrations of succinate and fumarate required to produce half-maximal reduction are 120 μM succinate in the presence of 24 mM fumarate, and 20 mM fumarate in the presence of 68 μM succinate. The two ratios of fumarate to succinate concentration that give 50 per cent oxidation-reduction of cytochrome *b* are 200 and 290, respectively.

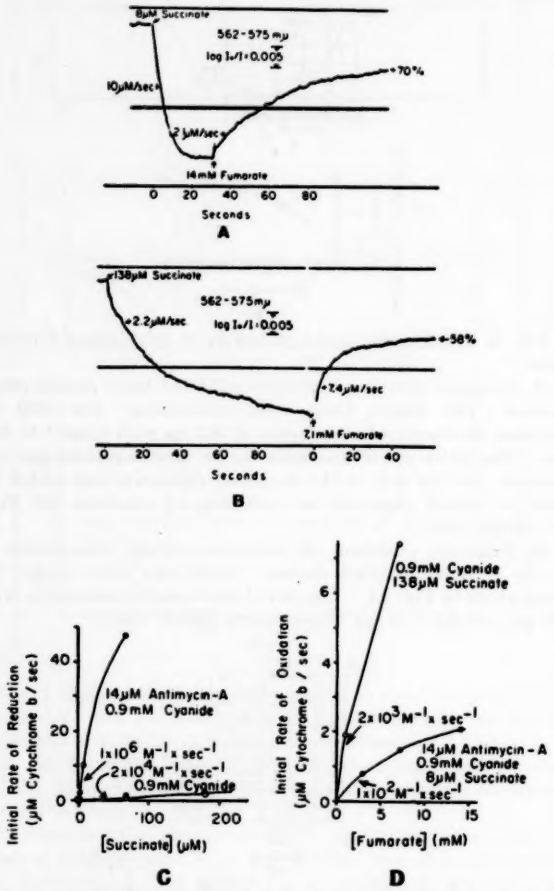


FIG. 4. The kinetics of the reduction of cytochrome *b* by succinate and its oxidation by fumarate in the presence and absence of antimycin A.

A, recordings (obtained with the double beam spectrophotometer) of the kinetics of reduction of cytochrome *b* in material treated with antimycin A. The material pretreated with 14 μM antimycin A and 0.9 mM cyanide is reduced upon the addition of 8 μM succinate. After the reaction is complete, the oxidation by fumarate is initiated (Experiment 286e-21).

B, reduction of cytochrome *b* in the cyanide-inhibited system. The heart muscle particles are pretreated with 0.9 mM cyanide, and 138 μM succinate is added. The oxidation reaction is caused by the addition of 7.1 mM fumarate. In both *A* and *B* the reaction rates are calculated from the slopes of the kinetics on the assumption that the molecular extinction coefficient of cytochrome *b* is 20 cm.$^{-1}$ mM^{-1} (Experiment 286-10).

C, effect of antimycin A on the kinetics of reduction of cytochrome *b* in cyanide-inhibited heart muscle preparation (Experiment 286e).

D, effect of antimycin A upon the kinetics of oxidation of cytochrome *b* in the cyanide-inhibited system. In this case, the concentrations of succinate present are similar to those used in obtaining *Traces A* and *B* (Experiment 286e).

To explain this significant change in the apparent equilibrium constant caused by treatment with antimycin A, we have measured the kinetics of cytochrome *b* reduction by succinate and of oxidation by fumarate in the presence and absence of antimycin A. The reduction of cytochrome *b* by the addition of 138 μM succinate to a cyanide-inhibited heart muscle preparation is illustrated in Fig. 4*B*. In agreement with results presented in Fig. 2 and with our findings in previous studies (2) concerning the sluggish response of cytochrome *b* of these particles, the reaction proceeds slowly to an end point. Even in the initial phases a maximal rate of only 4.5 μM cytochrome *b* per second is attained. Upon addition of fumarate, a relatively rapid oxidation takes place at an initial rate of 7.4 μM cytochrome *b* per second, and 50 per cent oxidation is obtained with a fumarate to succinate ratio of 54.

If, however, the heart muscle particles are pretreated with antimycin A (Fig. 4*A*), only 8 μM succinate is required for cytochrome *b* reduction at the rate of 10 μM per second, and the extent of reaction is even greater than that shown in Fig. 4*B*. In this case, oxidation of cytochrome *b* by fumarate is slow (1.7 μM cytochrome *b* per second), 14 μM fumarate being required to reach 70 per cent oxidation (6 μM for 50 per cent oxidation). It is clear from these two records that treatment with antimycin A has completely altered the equilibrium and kinetic properties of cytochrome *b*.

The effect of varying succinate and fumarate concentrations upon the rates of the reactions is illustrated by the graphs presented in Figs. 4*C* and 4*D*.[5] In the absence of antimycin A the rate of increase in reaction speed with succinate concentration is small; the slope of the curve corresponds to a second order velocity constant of 2×10^4 M^{-1} × sec.$^{-1}$ for the reduction of cytochrome *b* by succinate in the presence of cyanide. The slope of the line is about 50 times greater in the presence of antimycin A, and in its initial portion corresponds to a second order velocity constant of 10^6 × M^{-1} × sec.$^{-1}$ As might be expected, the line is not straight, because at higher succinate concentrations the rate of reduction of succinic dehydrogenase by succinate becomes limiting. In Fig. 4*D* similar data are plotted for the oxidation of succinate-reduced cytochrome *b* by fumarate. In this case, however, the scales of the *ordinate* and *abscissa* have been changed, respectively, 10- and 80-fold, since the oxidation of re-

[5] In these studies, initial slope is measured as soon as the disturbance caused by adding the reagent has subsided. As illustrated by the records presented in Fig. 4, this measurement rate can be made before a substantial extent of reaction has occurred.

duced cytochrome *b* by fumarate is much slower than the reduction of oxidized cytochrome *b* by succinate. In the absence of antimycin A the rate of oxidation of reduced cytochrome *b* by fumarate has an initial slope of about 2×10^3 M^{-1} $\times$ sec.$^{-1}$, whereas in the presence of antimycin A, the reaction is inhibited and the slope is only about 1×10^2 M^{-1} $\times$ sec.$^{-1}$ In both cases there is a considerable inflection of the curves, which suggests that the fumarate-hydrogenase activity is rate-limiting at higher concentrations of fumarate. In summary, treatment with antimycin A increases the second order velocity constant for reduction of cytochrome *b* by a factor of 50, and it decreases the corresponding constant for fumarate oxidation of cytochrome *b* by a factor of about 20. It is of interest to note that antimycin A inhibition appears to limit the maximal rate of the fumarate oxidation reaction.

DISCUSSION

General Features of Difference Spectra—Three features of these data concerning oxidized minus reduced spectra are noteworthy:

1. They support the previously reported result (12) that the cytochrome *c* content of the heart muscle preparation varies considerably, and with little change of oxidase activity, because cytochrome *c* is not rate-limiting in these preparations. The displacement of the absorption band of cytochrome *c* of the heart muscle preparation to wave lengths of 550 to 551 mμ, or higher, is attributable to cytochrome c_1, (10, 21). This preparation, together with the "low cytochrome *c*" preparation (Paper II, Fig. 3*C* (12)) and the washed mitochondrial preparations, shows clearly the cytochrome c_1 band at 553 mμ. Cytochrome *c* can scarcely be discerned at room temperature, but spectra measured at liquid air temperatures clearly identify cytochrome *c* as a component of the system.

2. The difference spectrum of the heart muscle preparation (Fig. 1*A*) shows absorbancy changes attributable to flavoprotein and, if the flavoprotein content is computed on the same basis as the analogous computation of the flavoprotein content of mitochondria (16, 17), enough is present to provide at least 1 mole of flavoprotein per mole of cytochrome *a*, as was assumed previously by Slater (22). As in the case of the liver mitochondria, some flavoprotein is reduced by succinate on treatment with antimycin A, more is reduced in anaerobiosis, and an additional portion with dithionite. The first portion probably represents flavoprotein associated with succinic dehydrogenase. This flavoprotein is rapidly reduced upon the addition of succinate, in contrast to the rather slow response of flavin in some of the purified preparations (23). The flavoproteins of the heart muscle preparation have spectroscopic characteristics similar to those in mitochondria, the difference spectrum showing a pronounced trough at 455 mμ and no corresponding trough in the vicinity of 370 mμ, as is found with most isolated flavoproteins.

3. The absorption spectrum indicates that cytochromes and other pigments of the heart muscle preparation in the vicinity of 340 mμ do not interfere with pyridine nucleotide measurement in the intact mitochondria or sarcosomes, although the absorption in the vicinity of 305 mμ is appreciable and could be partly responsible for the results obtained by Holton *et al.* (24) with heart sarcosomes.

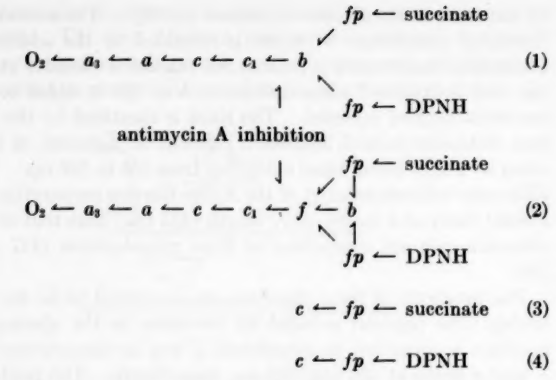
The "b" Pigments—By differential chemical treatments spectra are obtained that correspond to three "*b*" pigments. The first is cytochrome *b* which is completely, though slowly, reduced by succinate in the absence of oxidase activity. The second is a "modified cytochrome *b*" which is identified by the additional absorption band having a peak at 566 mμ and a shoulder at 560 mμ that is produced when antimycin A or QO is added to the succinate-reduced material. The third is identified by the fact that dithionite reduces additional pigment or pigments, as indicated by a very broad band extending from 556 to 566 mμ. The dithionite-reduced material of the Keilin-Hartree preparation has a Soret band at a longer wave length (435 mμ) than that of the dithionite-reduced component of liver mitochondria (427 mμ) (16).

The functions of these pigments are concluded to be the following: The pigment reduced by succinate in the absence of inhibitor is identified as cytochrome *b* and is characterized by γ- and α-peaks at 430 and 562 mμ, respectively. The positions of the bands are close to those reported by Sekuzu and Okunuki (25), but our data show a sharper α-band than that obtained by them. Neither the second nor the third component fulfills the requirements for respiratory carriers in succinate oxidation, since they cannot be reduced by this substrate in the absence of oxidase activity. The amount of the 566-mμ material relative to cytochrome *b* (562 mμ), as assayed in the manner indicated in Fig. 1*C*, gives results in the range of 30 to 70 per cent for various Keilin and Hartree preparations. Since the 566-mμ material is not a respiratory carrier, the succinic oxidase activity is not altered by preparations having a high content of it.

The clear-cut difference between the succinate- and dithionite-reduced pigments of the heart muscle preparation (*cf.* (12)) raises the question of the validity of studies of the oxidation-reduction potential of "cytochrome *b*" in which the dithionite-reduced pigment is taken as the end point. The value found (20) (−40 mv.) is probably much lower than the true value (see below for further discussion).

In a preliminary report (2) it was noted that the absorption band in the region of 555 to 570 mμ, obtained in the anaerobic succinate-reduced preparation, is more intense when antimycin A is present. The additional absorption was tentatively attributed to Slater's factor (26) or to a cytochrome *b*-antimycin A compound. Subsequently, studies of mitochondria, fly sarcosomes, and so forth, have shown that the intensified absorption attributable to antimycin A is not regularly measurable; thus neither hypothesis seems applicable. It now appears that the additional absorption resulting from antimycin A treatment, here shown to have a 566-mμ peak, is due to an inactive form of cytochrome *b*, *i.e.* one that can be activated only by the more rapid electron transfer to cytochrome *b* (562 mμ) which we have shown to occur in the presence of antimycin A.

Interaction of Cytochrome b with Flavoprotein and Cytochrome c_1—A consideration of the two mechanisms for electron transfer in phosphorylating and nonphosphorylating systems provides a basis for the explanation of the effects of the kinetics and equilibrium of cytochrome *b* reduction. In considering the mechanisms below, it should be remembered that in the nonphosphorylating DPNH and succinic oxidase system, cytochrome *b* is not reduced rapidly enough to be considered an active participant in electron transport. On the other hand, in the phosphorylating system, the kinetics of cytochrome *b* justify its inclusion in the electron transport chain.



In the phosphorylating system (Equation 1), flavoprotein transfers electrons readily to cytochrome *b* and does not bypass it. Disruption of the mitochondria usually leads to a loss of phosphorylative activity and to a change in the pathway of electron transfer to one that bypasses cytochrome *b* (Equation 2). The system is antimycin A-sensitive, and the site of action of the inhibitor is between cytochrome c_1 and a hypothetical factor (26). The Keilin and Hartree preparations behave in this way. Further purification leads insensitivity to antimycin A. The flavoprotein then interacts with cytochrome *c* without the intervention of cytochromes *b* or c_1; this is the cytochrome *c*-reductase activity of succinate or DPNH dehydrogenases (Equations 3 and 4). A related example of a change of flavoprotein specificity with purification is afforded by butyryl-CoA dehydrogenase (for a summary, see (27)). As an explanation for this stepwise loss of function of cytochrome *b*, we propose that the flavoprotein of the respiratory chain has a potentiality of cytochrome *c*-reductase activity which is not normally exhibited because cytochrome *c* is not directly accessible. During disruption of the mitochondria a series of structural alterations occurs in which portions of the chain, such as cytochromes c_1 and *c*, become accessible to the flavoprotein, resulting in a bypassing of cytochrome *b*. Extraction of the flavoproteins from the respiratory chain allows their direct interaction with cytochrome *c* to be demonstrated.

Kinetic studies of the Keilin and Hartree preparation show that cytochrome *b* is somewhat more responsive to electrons donated by succinic dehydrogenase than to those donated by DPNH dehydrogenase. This result suggests a different sensitivity of these two dehydrogenases to structural alterations (28).

Although some increase in the rate of cytochrome *b* reduction would be expected in the material treated with QO, because cytochrome c_1 no longer accepts electrons, the fact that the initial rate of cytochrome *b* reduction is greatly accelerated is of considerable importance. Since we have found experimentally that the rate of oxidation of cytochrome *b* is relatively slow (29), any great change in the initial rate, upon addition of the inhibitor, is unexpected. It seems necessary to postulate that the inhibitor alters the system so that the electrons that flow to cytochrome c_1 in the absence of the inhibitor are routed to cytochrome *b* in the presence of the inhibitor. Only by such a change can cytochrome *b* acquire such an increase over its initial rate of reduction in the presence of the inhibitor. A possible mechanism is suggested by Equation 2: that the $fp \rightarrow b$ interaction is enhanced by treatment with an inhibitor, and that, insofar as cytochrome *b* is concerned, the system reacquires a rapid reduction of cytochrome *b* and simulates the intact system of Equation 1. It is possible that many degrees of inactivation of cytochrome *b* can be obtained by a variation of the $fp \rightarrow b$ interaction in the absence of the inhibitor.

Jackson and Lightbown (30) have again taken up the proposal of Keilin (31) that cytochrome *b* is auto-oxidizable and, in the absence of these kinetic data, have proposed that a more rapid reduction of cytochrome *b* in the presence of QO would be the result of an inhibition of the auto-oxidation reaction. This simple "inhibition of auto-oxidation" theory is inadequate for the same reason as is the theory of inhibition of oxidation through the cytochrome chain, discussed above. Another objection to the explanation presented by these authors is the lack of evidence of the direct reaction of cytochrome *b* with oxygen at a significant rate.

The possibility that the inhibitor actually supplies a cofactor lacking in electron transfer from flavoprotein, however unlikely, is not to be ignored. This requires two modes of action for the inhibitor; first, to inhibit electron transfer between flavoprotein and cytochrome c_1, and second, to supply a cofactor acting between flavoprotein and cytochrome *b*.

These results shed some light on the nature of the cytochrome *b* bypass in nonphosphorylating systems: (*a*) cytochrome *b* itself can still accept electrons at a rapid rate when QO is present, *i.e.* cytochrome *b* itself is not inactivated; (*b*) flavoprotein can still donate electrons to cytochrome *b* at a rapid rate when QO is present. It would appear that disintegration of the mitochondria and the consequent loss of phosphorylation enhance the interaction of flavoprotein and cytochromes *c* and c_1 at the expense of the interaction of flavoprotein and cytochrome *b*.

Equilibrium and Kinetic Data—There are significant inconsistencies between the apparent equilibrium constants of the succinate-cytochrome *b* reaction computed from the titration data and those computed from the kinetic data. In the absence of antimycin A an apparent equilibrium constant (succinate to fumarate ratio) of 40 is suggested by the data presented in Fig. 2*B*, whereas the kinetic data in Fig. 4 show the ratio of second order velocity constants to be 10:1. In the material treated with antimycin A the discrepancy is much larger, for here the titration data (*cf.* Fig. 3*B*) give a ratio of 250:1, whereas the kinetic data give a ratio of 10,000:1, a 40-fold discrepancy.

The discrepancies between the titration and kinetic data suggest that we are not dealing with an equilibrium system and that different pathways exist for the succinate and fumarate reactions in the heart muscle preparation. The data of Singer *et al.* (23) have shown that the transfer of electrons from succinate to dyes is *o*-phenanthroline-sensitive and that this is not the case with the fumaric hydrogenase activity. The alternative hypothesis, that an equilibrium does exist and that the addition of antimycin A raises the oxidation-reduction potential of cytochrome *b* by over 0.1 volt is unlikely, especially since cytochrome *b* does not become reducible by ascorbate in the antimycin A-treated system.

The possibility of a nonequilibrium system indicates that Ball's data on the oxidation-reduction potential of cytochrome *b* should be accepted with caution and that the ferri-ferro oxalate titration of Hill (32), which gives $E'_0 = 0.0$ at pH 7.0 for the potential of cytochrome *b*, should be considered a more reliable value.

Slater (33) finds fault with our conclusions regarding the by-

passing of cytochrome *b* in nonphosphorylating preparations and attributes the slow reduction of this component in the aerobic-anaerobic transition to a slow approach to equilibrium in the presence of fumarate accumulated during succinate oxidation. Here we find experimentally that the succinate-fumarate ratio that Slater assumes (0.15) would give a 150:1 ratio for the rate of reduction to rate of oxidation. Thus the oxidation rate is negligible and the objection has no foundation.

SUMMARY

The spectroscopic, equilibrium, and kinetic properties of cytochrome *b* of the Keilin and Hartree succinic oxidase preparation have been investigated. Two chemical treatments for obtaining the difference spectrum of this component are discussed, both involving addition of succinate to (*a*) the ascorbate-reduced material or (*b*) the material treated with antimycin A. In the latter case an absorption band in addition to that of cytochrome *b* is found. This new band has a peak at 566 mμ and a small shoulder at 560 mμ. Since this 566-mμ component is not reduced by succinate without antimycin A treatment, it is attributed to a modified cytochrome *b*. Although in some Keilin and Hartree preparations there is roughly as much modified cytochrome *b* with a peak at 566 mμ (α-band) as there is cytochrome *b* with a peak at 562 mμ, their succinic oxidase activity is not impaired because neither cytochrome *b* nor the modified component participates in the electron transport. Pretreatment of the heart muscle preparation with antimycin A or 2-*n*-heptyl-4-hydroxyquinoline N-oxide drastically alters the kinetics and equilibrium of the reactions of cytochrome *b* with succinate and fumarate, the reduction by succinate being greatly accelerated and the oxidation by fumarate being inhibited. This acceleration of electron transfer between flavoprotein and cytochrome *b* in the presence of antimycin A or 2-*n*-heptyl-4-hydroxyquinoline N-oxide simulates a reconstitution of the electron-transfer activity of cytochrome *b* in the particles. It appears that the disruption of mitochondria involving a loss of phosphorylative activities favors the interaction of flavoprotein with cytochromes *c* and c_1 in preference to that with cytochrome *b*. Discrepancies between the kinetic and equilibrium data, especially in the presence of antimycin A, indicate that cytochrome *b* is not in reversible equilibrium with the succinate-fumarate system and that determinations of the oxidation-reduction potential of cytochrome *b* with this couple are open to question.

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Catalytic Oxidation of Glutathione and Other Sulfhydryl Compounds by Selenite

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(Received for publication, April 9, 1958)

In studies of the oxidation of sulfhydryl compounds (1), selenite has been found to be an active catalyst. Considering the biological importance of sulfhydryl oxidations and the implication of sulfhydryl compounds in selenium poisoning (2–5), more information on selenite-catalyzed oxidations is desirable. This paper reports studies of the catalytic oxidation of GSH by selenite and its reaction mechanism.

EXPERIMENTAL

Oxygen absorption was measured manometrically. After equilibration to 37° the manometer was closed to the atmosphere and the reaction was initiated by adding the solutions of catalyst and buffer from the side arms to the solution of sulfhydryl compound in the flask. All chemicals except CoA were analytical reagent or c.p. grade. The cysteine hydrochloride was purchased from the Fisher Scientific Company, GSH from Schwarz Laboratories, and CoA (approximately 75 per cent) from the Nutritional Biochemicals Corporation. Cysteine and GSH were chromatographically pure. Dihydrolipoic acid was a gift from Dr. J. A. Brockman of the American Cyanamid Company. Water was twice distilled in glass. Selenite and GSH were allowed to react by the method of Klug *et al.* (6) and the products were chromatographed on paper with the use of *n*-butanol-acetic acid-water, 50:25:25 by volume.

RESULTS

Catalysis of Oxidation of Sulfhydryl Compounds by Selenite and Other Metallic Salts—After preliminary comparisons of the catalytic activity of various metallic compounds, Na_2SeO_3, $CuCl_2$, $FeCl_3$, $MnSO_4$, and $CoCl_2$ were found to be the most active catalysts for the oxidation of sulfhydryl compounds. Results for the catalytic oxidation of cysteine, GSH, CoA, and dihydrolipoic acid are presented in Table I. The initial rate of cysteine oxidation was greater for Fe^{+++} catalysis than for Cr^{++} catalysis (Table I); however, after 10 minutes, the rates for Fe^{+++} and Cu^{++} were 8.0 μl. of O_2 per minute and 12.6 μl. of O_2 per minute, respectively. Selenate and selenite were equally active catalysts for GSH oxidation. For GSH oxidation as given in Table I, selenate catalysis gave an initial rate of 19.1 μl. of O_2 per minute compared to 18.2 μl. of O_2 per minute for selenite.

Effect of Selenite Concentration, pH, and Temperature—Study of the catalytic oxidation of GSH over a wide range of selenite concentration showed (Table II) that the rate of oxidation was a function of selenite concentration up to 0.01 mole of selenite per mole of GSH. Loss of catalytic activity at the higher concentrations of selenite was caused by its reduction to metallic selenium which is not a catalyst. This formation of metallic selenium is readily seen as a brown color and suspended red particles. The data in Table III show that the oxidation of GSH by selenite increases with increasing pH, and it is inhibited at pH 4.2. At higher pH values, above 8.4, the rate of the oxidation decreases because of the formation of metallic selenium which is inactive as a catalyst.

The rate of oxidation increased as a function of temperature. GSH of 4.9×10^{-5} M in 0.1 M phosphate buffer, pH 7.1, was catalytically oxidized by 0.01 mole of selenite per mole of GSH at rates of 9.0, 13.0, and 18.0 μl. of O_2 per minute at temperatures of 17°, 27°, and 37°, respectively. From the Arrhenius relationship, the activation energy was found to be 5.9 kilocalories per mole. In a similar reaction system, 0.01 mole of $CuCl_2$ per mole of GSH gave oxidation rates of 0.4, 0.9, and 1.7 μl. of O_2 per minute at temperatures of 17°, 27°, and 37°. The higher activation energy of 12.2 kilocalories per mole for Cu^{++} indicates that it is not as good a catalyst as selenite.

Inhibition of Selenite Catalysis—Tellurite and sulfite, salts of members of Group VI in the periodic table with selenium, were evaluated as inhibitors of selenite catalysis. Tellurite at equimolar concentrations was a good inhibitor (Table IV). Considering the chemical similarities, tellurite might compete directly with selenite. Tellurite also catalytically oxidized GSH, but its activity was much less than that of selenite. Sulfite did not inhibit selenite catalysis or catalyze GSH oxidation. When arsenite was allowed to react with GSH for 30 minutes before the addition of selenite, GSH oxidation was inhibited (Table V.) However, when arsenite was first added to selenite before the addition of GSH, there was little inhibition of the oxidation. A compound of arsenite and GSH might be the active inhibitor of selenite catalysis. The mechanism of this inhibition was not studied in detail.

Studies of Active Compound and Product Formed from Glutathione and Selenite—Chromatography of the products of the reaction of 1 mole of selenite and 4 moles of GSH in dilute HCl solution showed two components (Fig. 1). After ninhydrin treatment, the GSSG spot was purple and the spot for GS-Se-SG was greenish purple. This result is in agreement with that of Petersen (7) who separated the reaction products of GSH and selenious acid into components corresponding to GSSG and GS-Se-SG with the use of starch chromatography. It is also in accord with the views of Klug and Petersen (8) that the reaction of 1 mole of selenious acid with 4 moles of cysteine gives cystine and cysteine-Se-cysteine.

GS-Se-SG prepared by the method of Klug *et al.* (6) was compared as a catalyst for GSH oxidation with 0.01 mole of selenite per mole of GSH. On an equivalent selenium basis, GS-Se-SG

TABLE I

Effect of metal catalysts on rate of oxidation of sulfhydryl compounds

Sulfhydryl compound	Phosphate buffer	pH	Initial rate for catalysts at 0.01 mole per mole of sulfhydryl compound					
			$CuCl_2$	$FeCl_3$	$CoCl_3$	$MnSO_4$	Na_2SeO_3	No catalyst
	M		*μl. O_2/min.*					
4.89×10^{-5} M cysteine	0.1	7.23	16.3	21.1	2.1	1.0	14.0	0.4
4.89×10^{-5} M GSH	0.1	7.26	2.4	0.9	2.3	0.6	18.2	0.3
9.61×10^{-6} M dihydrolipoic acid	0.21	7.00	0	0	4.6	1.5	4.0	0
7.85×10^{-6} M CoA	0.14	7.38	0.9	0.3	0.5	0.3	1.1	0.2

TABLE II

Effect of concentration of selenite on oxidation of GSH

Concentration	Initial	Color of reaction mixture
mole Na_2SeO_3/mole GSH	*μl. O_2/min.*	
0	0.3	
0.0025	4.0	
0.0050	11.0	
0.010	18.0	Very pale yellow
0.10	10.0	Yellow, red particles
0.20	0.8	Brown, red particles
0.40	0.0	Brown, red particles

TABLE III

*Effect of pH on selenite catalysis of GSH oxidation**

pH (0.1 M buffer)	Rate of oxygen absorption	
	Initial	After 10 min.
	μl.O_2/min.	
4.19 (Phosphate)	0	0
6.60 (Phosphate)	13.0	3.9
7.18 (Phosphate)	18.0	6.8
8.38 (Tris)†	18.0	12.0
8.89 (Borate)	13.0	9.6
9.56 (Borate)	8.8	6.7

*The catalyst was 0.01 mole of selenite per mole of GSH.
†Tris = tris(hydroxymethyl)aminomethane.

TABLE IV

*Inhibition of selenite catalysis by tellurite**

Selenite concentration	Tellurite concentration	Initial rate
mole/mole GSH		*μl./O_2/min.*
0.01	0	18.0
0.01	0.01	0.8
0.01	0.05	1.8
0	0.01	0.8
0	0.05	4.5

* Selenite and tellurite were first mixed together in the side arm of the flask, and then they were added to the flask containing the GSH solution to initiate the oxidation.

and selenite were equally good catalysts, giving identical rates of 18 μl. O_2 per minute. Chromatographic separation (Fig. 1) of a sample taken during oxidation of GSH by selenite catalysis gave spots for GSSG and GS-Se-SG.

TABLE V

*Effect of arsenite on rate of oxidation of GSH**

Method of adding arsenite	Arsenite	Initial rate
	mole/mole GSH	*μl.O_2/min.*
$NaAsO_2$ + GSH	0	18.2
	0.025	9.9
	0.050	1.7
	0.10	0.8
	0.20	0.8
$NaAsO_2$ + Na_2SeO_3	0.025	17.6
	0.05	16.8
	0.10	15.3
	0.20	13.4

* The concentration of selenite was 0.01 mole of selenite per mole of GSH in every reaction.

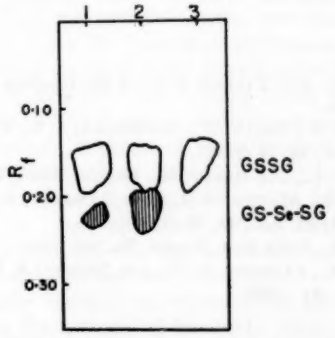

FIG. 1. Diagrammatic representation of the paper chromatograms showing GSSG and GS-Se-SG. *Column 1*, the sample taken during selenite-catalyzed GSH oxidation; *Column 2*, products of the reaction of selenite and GSH; *Column 3*, pure GSSG.

Similarly, by paper chromatography the final product of the oxidation was identified as GSSG. The stoichiometry of oxygen absorption showing an uptake of 0.87 mole of O_2 per 4 moles of GSH is in agreement with this result. Mixing pure GSSG with selenite and oxygen gave no further reaction.

DISCUSSION

The catalytic oxidation of cysteine by Cu^{++} and Fe^{+++} has been extensively studied, especially by Warburg (9). Comparison of selenite with Cu^{++} and Fe^{+++} shows that it is also a good catalyst for cysteine oxidation. Before this study was undertaken, Cu^{++} was known as the best catalyst for GSH oxidation (10, 11).

Thus, it was interesting to find that selenite was a far better catalyst.

Although it is well known that CoA and dihydrolipoic acid will undergo oxidation in a neutral or alkaline solution in the presence of heavy metals, this is the first intercomparison with other sulfhydryl compounds and a number of metal ions. In this comparison selenite was found to be a good catalyst for dihydrolipoic acid and CoA oxidation.

Because selenite is a most active catalyst for GSH oxidation, it is interesting to consider this reaction further. Painter (12) proposed that the general reaction between sulfhydryl compounds and selenious acid is as shown in Diagram 1.

$$4RSH + H_2SeO_3 \rightarrow RSSR + RS\text{-}Se\text{-}SR + 3H_2O$$
$$\downarrow$$
$$RSSR + Se$$

DIAGRAM I

Klug and Petersen (8) and Petersen (7) found this reaction sequence to apply in reactions of cysteine and GSH with selenious acid. Although this reaction sequence accounts for the oxidation of 4 moles of GSH per mole of selenite, the mechanism for the oxidation of large amounts of GSH by molecular oxygen in the presence of catalytic amounts of selenite is unknown. From the results of this study showing that the catalytic oxidation of GSH by selenite proceeds through the formation of GS-Se-SG, an over-all reaction may be written (Equations 1 and 2).

$$4GSH + SeO_3^{--} \rightarrow GSSG + GS\text{-}Se\text{-}SG + 2OH^- + H_2O \quad (1)$$

$$2OH^- + GS\text{-}Se\text{-}SG + O_2 \rightarrow GSSG + SeO_3^{--} + H_2O \quad (2)$$

The ionic reaction in Equation 1 should proceed spontaneously. In Equation 2 the hydroxyl ion is not only a direct reactant but it can also increase the rate by ionization of the sulfhydryl group of GSH. Although Equation 2 represents an over-all reaction for GS-Se-SG oxidation the reaction mechanism must be more complex. The effect of pH on GSH oxidation is much too small for Equation 2 to be the rate-determining reaction.

Results of this study show some interesting correlations with the known biochemistry of selenium poisoning. After injection or oral ingestion of selenite, the experimental animal typically shows losses of GSH from the blood and organs (2–5). These decreases of GSH could be caused by its catalytic oxidation by selenite. Arsenite is effective in counteracting selenium poisoning (3), and this might be related to its inhibition of selenite-catalyzed GSH oxidation.

SUMMARY

1. Comparison with the action of Cu^{++}, Fe^{+++}, Co^{++}, and Mn^{++} showed that selenite was a good catalyst for the oxidation of cysteine, dihydrolipoic acid, and Coenzyme A, and was most active for glutathione oxidation.

2. Glutathione oxidation was a function of selenite concentration; the rate increased with increasing pH and the activation energy was 5.9 kilocalories per mole.

3. Tellurite and arsenite inhibited selenite catalysis.

4. Selenium diglutathione was an active intermediate in the oxidation.

Acknowledgment—The advice of Professor E. P. Painter is appreciated.

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The Requirement for Serum Albumin in Oxidative Phosphorylation of Flight Muscle Mitochondria

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(Received for publication, July 3, 1958)

Serum albumin was reported to be an essential component in the oxidative phosphorylation system of insect flight muscle mitochondria (1, 2). Additional experiments describing this requirement are presented in this paper. The data show the specificity of albumin and suggest the active site of the protein.

EXPERIMENTAL

Houseflies, *Musca domestica*, of both sexes from 4 to 7 days old were used. These were reared and maintained by the procedure described earlier (3). Flight muscle mitochondria, or sarcosomes, were isolated by the technique already reported (4, 5).

Oxygen consumption measurements were made in a conventional Warburg apparatus. The temperature was 25° and the gas phase was air. Inorganic phosphate was assayed by the method of Fiske and SubbaRow (6).

ADP and hexokinase (practical, type III) were purchased from the Sigma Chemical Company. BSA,[1] crystallized, was obtained from Pentex Inc. and Armour Laboratories. Human serum albumin was prepared by Cutter Laboratories. Bovine γ-globulin and β-lactoglobulin were obtained from Pentex Inc. Bovine hemoglobin (purified) and blood Fraction IV-1, prepared by Cohn's method (7), were supplied by Armour Laboratories. Ovalbumin (crystalline) and edestin were obtained from Mann Research Laboratories, Inc. and the Worthington Biochemical Corporation, respectively.

RESULTS

The requirement for BSA in oxidative phosphorylation by insect flight muscle mitochondria is evident from the data in Table I. Without BSA there was no net phosphorylation when the substrate was either α-ketoglutarate or succinate. In fact, because of dephosphorylation of added ADP (8), more inorganic phosphate was found at the end of the incubation period than had been placed in the reaction medium. When the substrate was α-glycerophosphate, some phosphorylation was noted in the absence of BSA, but this was considerably less than that obtained in its presence. As is also shown in Table I, BSA stimulated the oxidation of these substrates.

In the above experiments mitochondria were isolated in the standard medium of 0.25 M sucrose + 5 mM EDTA and BSA was added only to the reaction mixture. The use of BSA in the isolation medium was evaluated, and the data (Table II) indicate that although the protein had some effect here, its outstanding action, shown in Table I, was apparent only during the reaction period. Mitochondria isolated with a medium of sucrose + BSA had greater P:O values than did those with sucrose alone or with sucrose + the plasma substitute, polyvinyl pyrrolidone, but they were not as effective as mitochondria isolated with sucrose + EDTA. Maximal phosphorylation was found when the isolation medium contained sucrose + EDTA + BSA. When BSA was added with the mitochondria, supplementary albumin to a final concentration of 2 per cent was required for maximal phosphorylation. Other experiments showed that mitochondria isolated with 0.32 M sucrose + 10 mM EDTA had P:O values similar to those with the standard medium and that EDTA could not be replaced by tris(hydroxymethyl)aminomethane buffer.

The specificity of BSA in oxidative phosphorylation is shown in Table III. The data show that proteins differ in their ability to effect coupling. BSA and human serum albumin were the most active. Moderate levels of activity were found with ovalbumin, blood Fraction IV-1, red blood cell stroma, and β-lactoglobulin. Other proteins, such as bovine γ-globulin and gelatin, were completely inactive. The activity found in the various preparations from blood cannot be attributed to their contamination with albumin. Analysis[2] of blood Fraction IV-1, hemoglobin, and red blood cell stroma revealed only 1.0, 0.2, and 0.2 per cent albumin, respectively. These values are too small to account for the observed activities (1). Other nonprotein polymers, such as gum arabic and the plasma substitute, polyvinyl pyrrolidone, were inactive as couplers.

Lipides usually bound to crystalline serum albumin were not responsible for the activity of the BSA. BSA was extracted with isooctane (9) or with an acetic acid-isooctane mixture (10), and all activity remained with the protein. The lipide fraction was inactive. Furthermore, α-tocopherol failed to substitute for BSA. It was noted, however, that α-tocopherol had some effect on insect mitochondria, for in its presence oxidation of α-glycerophosphate, succinate, and α-ketoglutarate was increased slightly.

Ionophoresis of crystalline BSA failed to dissociate the activity from the albumin. As is shown in Fig. 1, BSA migrated homogeneously, and protein was distributed in 17 tubes. The albumin in each tube was recrystallized with ethanol and assayed. The specific activity of each fraction was essentially the same. No activity was found in collection tubes which did not contain protein, although other material, especially lipides, were separated from the protein.

[1] The abbreviations used are: BSA, bovine serum albumin; and EDTA, ethylenediaminetetraacetate.

[2] Albumin was determined immunologically by Dr. Ludwig Sternberger.

TABLE I

Requirement for BSA in oxidative phosphorylation by flight muscle mitochondria

The reaction mixture contained 20 μmoles of K phosphate buffer, pH 7.4; 20 μmoles of $MgCl_2$; 10 μmoles of ADP; 50 μmoles of glucose; 150 Kunitz-McDonald units of hexokinase; 2 per cent BSA, where indicated; and a 0.5 ml. suspension of mitochondria in 0.25 M sucrose + 0.005 M EDTA, pH 7.3 (approximately 8 to 10 mg. of protein per ml.). The quantities of substrate used in the respective experiments were as follows: α-glycerophosphate, 100 μmoles; succinate, 90 μmoles; and α-ketoglutarate, 30 μmoles. The total volume of the Warburg flask was made to 2.5 ml. with 0.25 M sucrose. Duration of the experiments was 30 minutes.

Substrate	BSA	ΔO	ΔP	P:O
		μatoms	*μmoles*	
α-Ketoglutarate	+	6.0	9.7	1.6
α-Ketoglutarate	−	4.7	+2.3	0.0
Succinate	+	6.6	6.4	1.0
Succinate	−	5 9	+2.3	0.0
α-Glycerophosphate	+	9.9	9.7	1.0
α-Glycerophosphate	−	7.6	1.5	0.2

TABLE II

Comparison of isolation media on oxidative phosphorylation

The reaction mixture was the same as described in Table I. Substrate was α-ketoglutarate and BSA was added to the reaction medium in all experiments. Sucrose concentration was 0.25 M.

Isolation medium	P:O
Sucrose	1.0
Sucrose + BSA (2.0%)	1.3
Sucrose + polyvinyl pyrrolidone (7.3%)	0.9
Sucrose + EDTA (5 mM)	1.4
Sucrose + EDTA (5 mM) + BSA (2.0%)	1.6

TABLE III

Specificity of BSA in oxidative phosphorylation by flight muscle mitochondria

The reaction mixture was the same as described in Table I. Substrate was α-ketoglutarate. Test substances were assayed at a final concentration of approximately 2 per cent.

Test substance	P:O
BSA	1.8
Human serum albumin	1.6
Ovalbumin	0.6
Bovine γ-globulin	0.0
Bovine hemoglobin	0.5
Blood Fraction IV-1	1.0
Red blood cell stroma	0.9
Chick plasma	0.7
Chick embryo juice	0.1
Edestin	0.3
β-Lactoglobulin	0.9
Gelatin	0.0
Gum arabic	0.0
Polyvinyl pyrrolidone	0.0

Additional experiments revealed that the BSA factor was heat-labile (Table IV). Albumin heated for 9 minutes at 72° partially lost its capacity to couple. This treatment, which alters the immunological behavior of serum albumin (11), did not affect its ability to stimulate oxidation. On the other hand, heating BSA at 100° for 15 minutes completely destroyed all activity. Not only was phosphorylation eliminated but respiration was no longer enhanced. Table IV also shows that subjecting BSA to alkali, pH 13.1, for 8 minutes, a condition which modifies the immunological properties of albumin (11), similarly lessened its capacity to effect phosphorylation.

The experiments described in Table V suggest the active site of the albumin. BSA was iodinated with 10 atoms of iodine per mole of protein (12). Assay of the iodinated albumin showed that its ability to couple phosphorylation was appreciably diminished. Since iodine caused both an oxidation of sulfhydryl groups and a conversion of tyrosyl residues to diiodotyrosyl residues, the effect of each modification was examined inde-

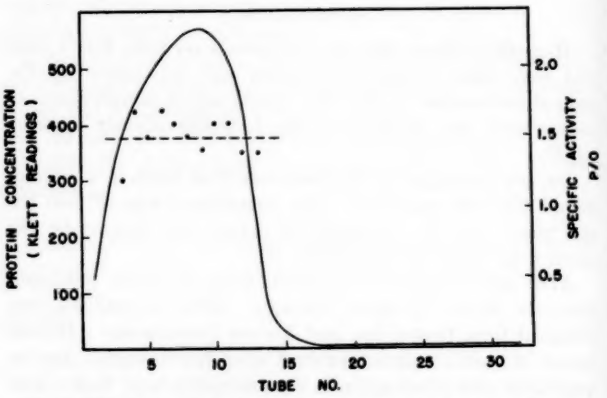

FIG. 1. Oxidative phosphorylation activity and the ionophoretic distribution of BSA. 100 ml. of 10 per cent albumin in 0.05 M borate buffer, pH 9.1, was ionophoresed with 600 volts at 25° using a Spinco model CP continuous flow paper electrophoresis apparatus. The distribution of protein, measured by phenol reagent, is shown as a continuous curve. The specific activity of each protein fraction is shown as a point. The dashed horizontal line represents the level of activity of the starting serum albumin.

TABLE IV

Modification of BSA and its ability to couple oxidation phosphorylation

Reaction mixture was the same as reported in Table I. The substrate was α-ketoglutarate. BSA was treated as indicated.

Experiment	ΔO	ΔP	P:O
	μatoms	*μmoles*	
No treatment	5.9	9.2	1.6
72° for 9 min.	5.8	6.3	1.1
No treatment	5.8	10.2	1.8
100° for 15 min.	4.4	+1.3	0.0
No treatment	5.4	9.3	1.7
pH 13.1 for 8 min	5.3	5.9	1.1

TABLE V

Effect of reaction of BSA with p-chloromercuribenzoate and iodine on its ability to couple oxidative phosphorylation

The reaction mixture was the same as reported in Table I. The substrate was α-ketoglutarate. For experimental details concerning treatment of BSA see the text.

BSA	P:O
Untreated	1.4
Iodinated	0.7
Reacted with *p*-chloromercuribenzoate	0.3
Reacted with *p*-chloromercuribenzoate then iodinated	0.2
Reacted with *p*-chloromercuribenzoate then iodinated, dialyzed versus cysteine	1.5

pendently. First, BSA was reacted with *p*-chloromercuribenzoate (13). After dialysis against buffer to remove uncombined *p*-chloromercuribenzoate, the albumin was tested and found to be largely inactive. This treated BSA was then iodinated so that now the tyrosyl groups were altered. When tested, the albumin was essentially inactive. The inactive protein was then dialyzed against cysteine which removed combined *p*-chloromercuribenzoate and restored the original sulfhydryl groups but kept tyrosyl groups iodinated. The albumin was now found to have its original coupling capacity. Other experiments showed that neither cysteine nor glutathione substituted for BSA.

DISCUSSION

The data show that plasma albumin was required for oxidative phosphorylation of flight muscle mitochondria. Other proteins varied in their ability to effect coupling and some were completely inactive. These differences together with those found by Pullman and Racker (14) and Polis and Shmukler (15) in related experiments on the reactivation of oxidative phosphorylation in aged mitochondria from mammalian liver make improbable the early viewpoint that BSA acted solely by providing a satisfactory osmolar environment which preserved the structural integrity of the mitochondria (16, 1).

The present results also show that BSA functions differently in the system examined here from the way it functions in the cytochrome *c* reductase system studied by Nason *et al.* (9). In activating the reductase, BSA was the carrier of the essential lipide, whereas in this study the lipides bound to BSA were inactive. On the other hand, the isooctane-extracted protein was fully active. Moreover, the ionophoretic distribution patterns for protein and activity were identical. These findings suggest that the BSA factor described in this paper is the albumin protein.

The need for BSA in phosphorylation of insect flight muscle mitochondria as described here and earlier (1, 2) has its parallel in the restoration of phosphorylation in aged mitochondria from mammalian liver (14, 15, 17, 18). With mammalian preparations a decrease in phosphorylation was associated with a release from the mitochondria of an inhibitory substance termed mitochrome (15). This protein, or a lipide bound to it (19), from aged mitochondria uncoupled oxidative phosphorylation of freshly prepared mitochondria. BSA reversed this inhibition. A similar explanation can account for the BSA need in oxidative phosphorylation with insect mitochondria. However, in contrast to the results with mammalian preparations, flight muscle mitochondria show a requirement for BSA even in the freshly prepared stage. This suggests that insect mitochondria release the phosphorylation inhibitor very quickly or that they are extremely sensitive to the uncoupler.

Polis and Shmukler showed an interaction between mitochrome and BSA (15). The present experiments establish the importance of the sulfhydryl moiety of BSA, which according to Boyer (13), is susceptible to combination with *p*-chloromercuribenzoate, in the coupling of oxidative phosphorylation. This may offer a clue to the nature of the phosphorylation inhibitor.

SUMMARY

Serum albumin was required in the oxidative phosphorylation system of insect flight muscle mitochondria. Without the protein there was little or no net phosphorylation and respiration was markedly lower.

Other proteins varied in their ability to effect coupling and some were completely inactive. This indicates that the albumin has a role other than that of merely providing a satisfactory osmolar environment for the mitochondria.

The activity of serum albumin was intimately associated with the albumin protein. A free sulfhydryl group of the albumin was necessary for oxidative phosphorylation. This may suggest the possible site of action between serum albumin and the phosphorylation inhibitor.

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Reductive Degradation of Pyrimidines

IV. PURIFICATION AND PROPERTIES OF DIHYDROURACIL HYDRASE*

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(Received for publication, July 23, 1958)

In previous papers in this series cell extracts of *Clostridium uracilicum* were shown to convert uracil to β-alanine, carbon dioxide, and ammonia with dihydrouracil and *N*-carbamoyl-β-alanine (β-ureidopropionic acid) as intermediates (1, 2). The properties of a partially purified enzyme from *C. uracilicum* that catalyze the conversion of uracil to dihydrouracil have also been described (3).

The purpose of this paper is to describe the purification and properties of the enzyme from *C. uracilicum* that are responsible for the conversion of dihydrouracil to *N*-carbamoyl-β-alanine. The enzyme has been named dihydrouracil hydrase (4).

EXPERIMENTAL

Materials—Uracil, thymine, and β-alanine were purchased from Nutritional Biochemicals Corporation. Dihydrouracil was synthesized as described earlier (2) and was later obtained from the California Foundation for Biochemical Research. *N*-Ethylmaleimide and *p*-chloromercuribenzoate were obtained from Mann Research Laboratories. *N*-carbamoyl-β-alanine was synthesized from β-alanine and potassium cyanate (5). Calcium phosphate gel was prepared according to Keilin and Hartree (6).

Methods—The methods of culture and preparation of cell-free extracts of *C. uracilicum*, strain M5-2, were described previously (2). *N*-carbamoyl-β-alanine was determined quantitatively with the use of the colorimetric procedure of Archibald (7). Dihydrouracil was determined as *N*-carbamoyl-β-alanine after hydrolysis with 0.1 N NaOH. Paper chromatography of reaction mixtures was carried out as described earlier (2, 8). Protein concentration was estimated by the method of Lowry *et al.* (9).

Assay of Dihydrouracil Hydrase—For assay purposes the reaction mixture contained 20 μmoles of dihydrouracil, 10 μmoles of $MgCl_2$, 200 μmoles of Tris,[1] pH 8.5, and the enzyme in a total volume of 3.0 ml. The reaction mixtures were incubated in glass-stoppered conical centrifuge tubes at 30° for 15 minutes. The incubation mixtures were deproteinized by the addition of 1.0 ml. of 10 per cent $HClO_4$. The precipitated protein was removed by centrifugation at 10,000 × *g* for 10 minutes, and the supernatant liquid was analyzed for *N*-carbamoyl-β-alanine. A unit of enzyme is defined as that amount of enzyme which forms 1 μmole of *N*-carbamoyl-β-alanine from dihydrouracil under the conditions of the standard assay given above; specific activity is expressed as units per mg. of protein.

Purification of Dihydrouracil Hydrase—All purification steps were performed at 0–5°. Centrifugations during fractionation were carried out at 10,000 × *g* for 10 minutes.

Removal of Nucleic Acid—Nucleic acid was removed from the cell-free extract by precipitation with $MnCl_2$ as described previously (3). The precipitate was removed by centrifugation and discarded. The supernatant liquid was dialyzed for 12 hours against 0.05 per cent sodium sulfide.

Ethanol Fraction—The dialyzed solution was adjusted to pH 6.5 with dilute acetic acid, and 2 volumes of cold (−10°) 95 per cent ethanol were added with stirring for 1 hour. The precipitate was removed by centrifugation and dissolved in 100 ml. of water.

First Ammonium Sulfate Fraction—To the ethanol fraction were added with stirring 45 gm. of solid ammonium sulfate. After the fraction was stirred for 20 minutes, the precipitate was removed by centrifugation and discarded. 20 gm. of ammonium sulfate were added to the supernatant solution with stirring for 20 minutes. The precipitate was removed and dissolved in 40 ml. of water.

Acetone Fraction—To the first ammonium sulfate fraction was added 1 volume of cold (−10°) acetone. The fraction was stirred for 30 minutes and the precipitate removed by centrifugation and discarded. To the supernatant liquid 1 volume of acetone was added. After the mixture had been stirred for 1 hour the precipitate was removed by centrifugation and dissolved in 20 ml. of water.

Calcium Phosphate Gel Treatment—Calcium phosphate gel (0.8 volume) was stirred into the solution (pH 6.5). The suspension was stirred for 20 minutes, centrifuged, and the gel discarded.

Second Ammonium Sulfate Fraction—To the supernatant fluid from the gel treatment were added 10 gm. of ammonium sulfate. After the mixture was stirred for 15 minutes the precipitate was removed and dissolved in 10 ml. of Tris buffer, pH 8.5. At this stage, the enzyme is stable for 6 months at −20°. Table I summarizes a typical purification procedure for the enzyme and shows an approximately 80-fold purification. This is in the range of purification usually obtained (60- to 100-fold) by the fractionation procedure used.

Specificity of the Enzyme—The enzyme was tested for activity

* Scientific Paper No. 1761 (Project 1351), Agricultural Experiment Stations, Institute of Agricultural Sciences, State College of Washington, Pullman, Washington. This investigation was supported in part by funds provided for biological and medical research by the State of Washington (Initiative No. 171).

[1] The abbreviation used is: Tris, tris(hydroxymethyl)aminomethane buffer.

TABLE I

Purification of dihydrouracil hydrase

Fraction	Volume of solution	Units	Specific activity	Yield
	ml.		*units/mg.*	%
Cell-free extract	400	26,000	12.5	
Dialyzed $MnCl_2$ supernatant fluid	450	18,400	24.7	71
Ethanol	100	16,900	95.6	65
First ammonium sulfate	40	14,040	265.8	54
Acetone	20	12,480	645.0	48
Calcium phosphate gel treatment	26	8,580	763.0	33
Second ammonium sulfate	10	4,680	995.0	18

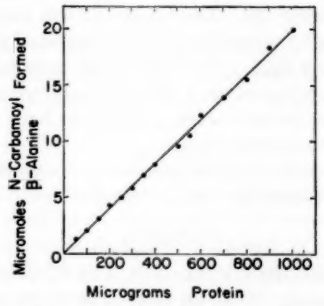

FIG. 1. Effect of enzyme concentration on rate of conversion of dihydrouracil to *N*-carbamoyl-β-alanine. The conditions of the standard assay are as described in the text.

with uracil, thymine, orotic acid, dihydrouracil, dihydrothymine, dihydroorotic acid, and hydantoin. No activity was observed with any of these compounds except dihydrouracil. The purified enzyme had no dihydrouracil dehydrogenase activity nor did it convert *N*-carbamoyl-β-alanine to β-alanine, carbon dioxide, and ammonia. The enzyme was also inactive in degrading carbamyl phosphate to carbon dioxide and ammonia.

Influence of Enzyme and Substrate Concentration on Rate of Conversion of Dihydrouracil to N-Carbamoyl-β-alanine—Under conditions of the standard assay the rate of dihydrouracil conversion to *N*-carbamoyl-β-alanine was proportional to the amount of enzyme present (Fig. 1). The rate of the conversion was also studied as a function of dihydrouracil concentration. The data were calculated graphically according to Lineweaver and Burk (10). The K_m was calculated to be 1.21×10^{-3} M.

Effect of Temperature on Enzyme Activity—Enzyme activity against dihydrouracil was measured over a temperature range of 20–50°. The enzyme had an optimal temperature range of 30–35° (Fig. 2). At 45° the enzyme was rapidly inactivated (Fig. 3).

Effect of pH on Enzyme Activity—The effect of pH was studied over the range of 6.0 to 10.5. Phosphate buffer was used for the range of pH 6.0 to 7.5, Tris buffer over the range 7.5 to 9.0, and carbonate-bicarbonate buffer from pH 9.0 to 10.5. The optimal pH range was 7.8 to 9.0. Activity fell off sharply on either side of this range (Fig. 4). For example, at pH 6.5 and 9.5 the relative activity was only 36 and 58 per cent, respectively, of that observed at pH 8.5.

Balance Data on Conversion of Dihydrouracil to N-Carbamoyl-β-alanine—Typical quantitative data on the conversion of dihydrouracil to *N*-carbamoyl-β-alanine are presented in Table II. It can be seen that there is fairly good agreement between the amount of dihydrouracil utilized and the amount of *N*-carbamoyl-β-alanine formed.

Effect of Dialysis and Versene on Enzyme Activity—Dialysis of the purified enzyme against tap water at 1° for 36 hours caused no appreciable loss of enzyme activity. Dialysis against distilled

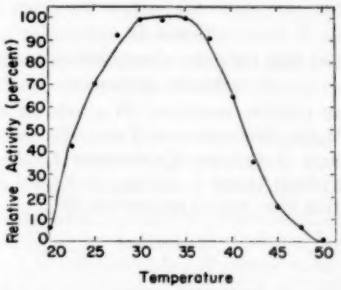

FIG. 2. Effect of temperature on dihydrouracil hydrase activity. The reaction mixtures contained 20 μmoles of dihydrouracil, 10 μmoles of $MgCl_2$, 200 μmoles of Tris Buffer, pH 8.5, and 0.65 mg. of enzyme in a total volume of 3.0 ml. The reaction time was 15 minutes.

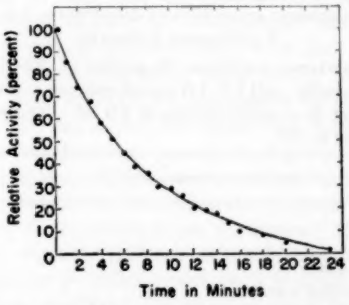

FIG. 3. Thermal stability of dihydrouracil hydrase at 45°. Samples of the purified enzyme were incubated at 45° in the absence of substrate. At suitable time intervals, aliquots were removed and their activities tested under conditions of the standard assay as described in the test.

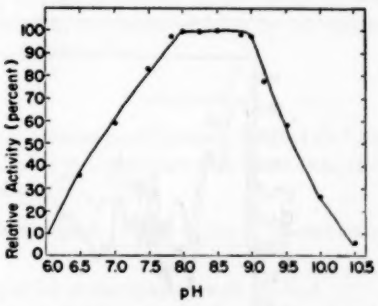

FIG. 4. Effect of pH on dihydrouracil hydrase activity. Reaction mixtures contained 20 μmoles of dihydrouracil, 10 μmoles of $MgCl_2$, 200 μmoles of the appropriate buffer (see the text) and 0.72 mg. of enzyme in a total volume of 3.0 ml. The reaction time was 20 minutes at 30°.

water for 12 hours, however, resulted in almost complete loss of activity when $MgCl_2$ was omitted from the assay system. Activity could be restored by the addition of 5 μmoles of Mg^{++} or Mn^{++} ion, either as the chloride or sulfate salt, to the reaction mixture. Treatment of undialyzed extracts with 1×10^{-3} M Versene (disodium ethylenediaminetetraacetate, Dow Chemical Company) caused a 96 per cent reduction in activity. The inhibition by Versene could be overcome by the addition of an excess of Mg^{++} or Mn^{++} (Table III). These data indicate that Mg^{++} or Mn^{++} ions are essential for the conversion of dihydrouracil to *N*-carbamoyl-β-alanine.

TABLE II

Balance data for conversion of dihydrouracil to N-carbamoyl-β-alanine

The reaction mixture contained 20 μmoles of dihydrouracil, 10 μmoles of $MgCl_2$, 200 μmoles of Tris buffer, pH 8.5, and the indicated amount of enzyme (Experiment 1, 250 μg.; Experiment 2, 500 μg.; Experiment 3, 1.0 mg.) in a total volume of 3.0 ml. The reaction time was 15 minutes at 30°.

Substrate	Experiment 1	Experiment 2	Experiment 3
	μmoles	μmoles	μmoles
−Dihydrouracil	5.2	9.64	18.5
+*N*-carbamoyl-β-alanine	4.9	10.31	20.2

TABLE III

Effect of dialysis, Versene treatment and Mg^{++} or Mn^{++} ions on enzymatic conversion of dihydrouracil to N-carbamoyl-β-alanine

Reaction mixtures contained 20 μmoles of dihydrouracil, 200 μmoles of Tris buffer, pH 8.5, 1.0 mg. of enzyme and the additions indicated below in a total volume of 3.0 ml. The reaction time was 20 minutes at 30°.

Enzyme treatment	Activity
	%
Undialyzed	100
Dialyzed, tapwater	97.4
Dialyzed, distilled water	6.5
Dialyzed, distilled water + 5 μmoles of Mg^{++}	96.4
Dialyzed, distilled water + 5 μmoles of Mn^{++}	91.8
Dialyzed, distilled water + 5 μmoles of Mg^{++} + 5 μmoles of Mn^{++}	97.3
Undialyzed + 1×10^{-3} M Versene	4.0
Undialyzed + 1×10^{-3} M Versene + 10 μmoles of Mg^{++} or Mn^{++}	96.8

Effect of N-Ethylmaleimide and p-Chloromercuribenzoate Enzyme Activity—The sulfhydryl inhibitors, *N*-ethylmaleimide and *p*-chloromercuribenzoate, had no effect on the activity of the enzyme even at a concentration of 1×10^{-2} M. The failure of these compounds to cause inhibition indicates that the enzyme does not require free sulfhydryl groups for activity.

Isolation and Identification of N-Carbamoyl-β-alanine as Conversion Product of Dihydrouracil—Paper chromatography of reaction mixtures (2, 8) revealed only one spot. Its chromatographic behavior, in the three solvent systems used, was identical with that of authentic *N*-carbamoyl-β-alanine.

To obtain a sufficient amount of the compound for identification, *N*-carbamoyl-β-alanine was isolated from large scale experiments by adsorption and elution from a Dowex 1-formate column as described earlier (2). The compound was isolated from the effluent fraction by crystallization from methanol at 0° and dried in a vacuum over P_2O_5. The yield of the recrystallized product was 22.5 mg., representing a 38.6 per cent recovery from dihydrouracil, the product being assumed to have the molecular weight of *N*-carbamoyl-β-alanine.

Elementary analysis of the compound as compared with that of *N*-carbamoyl-β-alanine was as follows:

$C_4H_7O_3N_2$

Calculated: C, 36.64 H, 5.34 N, 21.37
Found: C, 36.59 H, 5.16 N, 21.28

The product melted at 169° to 170° and a mixed melting point with authentic *N*-carbamoyl-β-alanine showed no depression. On evaporation of the product in 0.1 N HCl, a compound was formed which had the paper chromatographic properties of dihydrouracil (2).

The infrared absorption spectrum of the isolated product was determined and compared with that of authentic *N*-carbamoyl-β-alanine. Fig. 5 shows that the enzymatic product has the same infrared absorption spectrum as the authentic compound.

These data clearly establish that the enzymatic product of dihydrouracil hydrolysis is *N*-carbamoyl-β-alanine.

Enzymatic Conversion of N-Carbamoyl-β-alanine to Dihydrouracil—The reversibility of the conversion of dihydrouracil to *N*-carbamoyl-β-alanine was demonstrated by incubation of the enzyme with *N*-carbamoyl-β-alanine at pH 5.0. The rate of the reaction was measured by determination of the change in substrate concentration as a function of time (Fig. 6). Paper

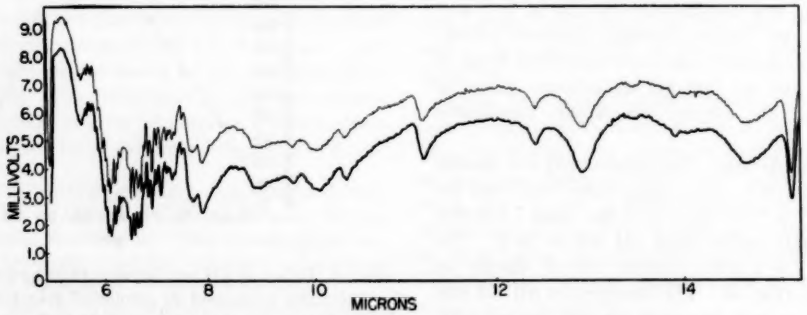

FIG. 5. Infrared spectra of authentic *N*-carbamoyl-β-alanine (*upper curve*) and the enzymatic product (*lower curve*) in KBr on a Perkin-Elmer recording spectrophotometer.

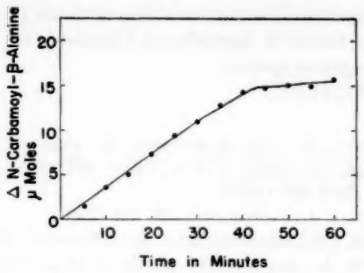

FIG. 6. Enzymatic conversion of *N*-carbamoyl-β-alanine to dihydrouracil. The reaction mixture contained 20 μmoles of *N*-carbamoyl-β-alanine, 250 μmoles of acetate buffer, pH 5.0, and 2.0 mg. of enzyme in a total volume of 5.0 ml. The incubation was at 30°.

chromatography of the reaction mixture after 40 minutes' incubation showed the presence of two components with properties identical to dihydrouracil and *N*-carbamoyl-β-alanine.

DISCUSSION

In a previous paper it was shown that the first step in the catabolism of uracil by *C. uracilicum* involves a reduced diphosphopyridine nucleotide-dependent reduction of uracil to dihydrouracil. Canellakis (11) has shown that the corresponding reduction of uracil and thymine to dihydropyrimidines is a triphosphopyridine nucleotide-dependent reaction with acetone powders of rat liver.

The purification of the enzyme from *C. uracilicum* which degrades dihydrouracil to *N*-carbamoyl-β-alanine provides additional evidence for the reductive pathway of pyrimidine catabolism by this organism. This enzyme, as well as dihydropyrimidine dehydrogenase, has also been purified from animal tissues and studied by Grisolia *et al.* (4, 12). The dihydrouracil hydrase of *C. uracilicum* differs in its substrate specificity from the calf liver enzyme in that it is specific for dihydrouracil, whereas the calf liver enzyme attacks dihydrouracil, dihydrothymine, and hydantoin (4). This is not too surprising when it is remembered that the bacterial enzyme is an induced enzyme synthesized only when uracil is present in substrate levels in the growth medium (2). Both the bacterial and the liver enzyme catalyze the interconversion of the appropriate dihydropyrimidines and the corresponding *N*-carbamoyl-β-amino acids, although the liver enzyme does not catalyze the conversion of *N*-carbamoyl-glycine to hydantoin (4).

From this and previous studies it appears that dihydropyrimidines are important intermediates in the catabolism of pyrimidines by a variety of biological systems. The dihydro compounds have been implicated in pyrimidine degradation in animal tissues (4, 11–18), in the intact rat (19), and in some microorganisms (2, 3, 20). Slotnick (21, 22) reported that mutant strains of *Escherichia coli*, originally characterized as requiring β-alanine or pantothenic acid for growth, could utilize dihydrouracil or *N*-carbamoyl-β-alanine for growth.

With the exception of dihydroorotic acid (23), the role of dihydropyrimidines in the synthesis of pyrimidine nucleotides has not been clearly established. Evidence against their direct conversion to mouse tumor nucleic acid has been presented by Lagerkvist *et al.* (24). Visser *et al.* (25) reported that neither dihydrocytidine phosphate nor dihyrouridine phosphate was incorporated into the RNA and DNA pyrimidines in the rat. Green and Cohen (26) have also shown that dihydrothymidine, dihydrodeoxyuridine, and dihydrouridine were inert to bacterial enzymes that catalyze nucleoside cleavage and pyrimidine exchange reactions. Slotnick and Weinfeld (22) have reported that dihydrouracil is not a precursor of nucleic acid pyrimidines in certain strains of *E. coli*. Cohen *et al.* (27) reported that dihydrouracil and dihydrocytosine, as the free base, riboside, or deoxyriboside could not replace the uracil, thymine, or cytosine requirement of pyrimidine mutant strains of *E. coli*. Similarly, dihydro-5-hydroxymethyluracil, as the free base, or deoxyriboside could not replace the thymine requirement of *E. coli* 15_{T-} (28).

Some evidence has been presented, however, which indicates that dihydropyrimidines or their derivatives may possibly have a role in nucleic acid metabolism. Green and Cohen (26) have reported the formation of dihydrodeoxyuridine nucleotide from dihydrodeoxyuridine by a thymine-requiring bacterium, indicating that phosphorylation of the dihydropyrimidine nucleosides can occur in living organisms. Grossman and Visser (29) have isolated dihydrocytidylic acid from liver, which fact indicates that the enzymatic reduction of pyrimidine nucleosides may occur naturally. Mokrasch and Grisolia (30) have evaluated the relative contribution of the orotic acid and the dihydropyrimidine pathways to RNA synthesis in several animal tissues. Evidence was presented showing that the intermediates related to the dihydropyrimidine pathway are incorporated into RNA. No direct interconversion between orotic acid and the dihydropyrimidine derivatives occurred, which seems to exclude the mediation of orotic acid in RNA synthesis in the tissues studied.

Canellakis (31) has shown that tissues that incorporate uracil into RNA have a decreased capacity to degrade this compound or the intermediates, dihydrouracil and *N*-carbamoyl-β-alanine. Whether dihydrouracil is involved in the synthesis of pyrimidine nucleotides by *C. uracilicum* is not yet known. However, since the first two enzymes involved in uracil degradation by this organism catalyze reversible reactions, it is possible that under the appropriate conditions dihydrouracil could be converted to uracil. Uracil could then be converted to pyrimidine nucleotides via uridine-5-phospate by condensing with 5-phosphoribosylpyrophosphate. Evidence has been presented for the occurrence of this reaction in *Lactobacillus bifidus*, (32), in mutant strains of *E. coli* (32, 35), in certain strains of *Lactobacillus bulgaricus* (33), and in the Ehrlich ascites tumor (34). Studies are now in progress to determine whether or not this reaction can take place in *C. uracilicum*.

SUMMARY

An enzyme, dihydrouracil hydrase, isolated and partially purified from extracts of *Clostridium uracilicum*, was shown to catalyze the reaction:

$$H_2O + \text{dihydrouracil} \underset{\longleftarrow}{\xrightarrow{Mg^{++} \text{ or } Mn^{++}}} N\text{-carbamoyl-}\beta\text{-alanine}$$

Certain properties of the enzyme were studied.

The enzymatic product of dihydrouracil conversion was isolated in crystalline form and identified as *N*-carbamoyl-β-alanine by the following criteria: elementary analysis, melting point determinations, paper and column chromatography, and infrared absorption spectrophotometry.

The role of dihydropyrimidines in the catabolism of pyrimidines and in the biosynthesis of pyrimidine nucleotides was discussed.

Acknowledgment—The author wishes to thank Dr. R. J. Foster of the Department of Agricultural Chemistry for running the infrared absorption spectra.

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Studies in Histochemistry

LI. MICRODETERMINATION OF HYALURONIDASE AND ITS INHIBITION BY FRACTIONS OF ISOLATED MAST CELLS*

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(Received for publication, June 16, 1958)

There should be considerable use for a procedure for the measurement of hyaluronidase in histologically or cytologically defined samples, *e.g.* microtome sections of tissue, small groups of isolated cells, or even single large cells such as protozoa. Adaptation of turbidimetric methods to a microscale was undertaken for this reason, as well as for the immediate need of a method for the determination of hyaluronidase inhibition by fractions of isolated mast cells.

Two turbidimetric procedures were tested, one based on the method of Tolksdorf *et al.* (1), which is one of a number of modifications of turbidimetric methods reviewed by Tolksdorf (2), and the other based on the more recent method of Di Ferrante (3). The Tolksdorf method proved preferable for inhibitor studies, and it was adapted to micromeasurements requiring samples 0.01 to 0.001 of the usual magnitude.

The Di Ferrante method was found to be unsuitable for the present inhibitor study, because the high concentration of NaCl used (0.15 M) interfered with the enzyme inhibition by heparin. Thus, barely perceptible inhibition was given by 40 μg. of heparin per ml. of reaction mixture, although 5 μg. per ml. gave 80 per cent inhibition by the Tolksdorf method. Interference of the heparin inhibition by NaCl is well known; it has been discussed, for example, by Meyer (4) and by Alburn *et al.* (5). Another difficulty arises when magnesium ions are added to obtain optimal inhibition by the nonspecific inhibitor of blood. The turbidity which is developed by the alkaline reagent is influenced by the precipitation of magnesium hydroxide.

Mast cells contain heparin material, and heparin is a heat-stable inhibitor of hyaluronidase. The possibility that the heat-labile nonspecific hyaluronidase inhibitor in blood serum is derived from the heparin-protein complex of mast cells was raised by Glick and Sylvén (6). Circumstantial evidence submitted by Glick and Ochs (7) seemed to be compatible with this possibility. Subsequently, Newman *et al.* (8) isolated from human blood plasma a carbohydrate-containing protein which was a hyaluronidase inhibitor with certain properties characteristic of the inhibitor of fresh plasma. The protein had 0.1 per cent sulfur as sulfate and therefore could not be a heparin-protein complex.

The possibilities remained that the protein described by Newman *et al.* is not the only heat-labile hyaluronidase inhibitor in serum or plasma and that mast cells contain not only heat-stable heparin material but also heat-labile hyaluronidase inhibitor which may be contributed to the blood. In this study a heat-labile inhibitor was not found in mast cells; in fact heating increased the inhibition by mast cell fractions. Reviews dealing with morphological and chemical constituents of mast cells have appeared recently (9–12).

EXPERIMENTAL AND RESULTS

Apparatus

The apparatus for the microadaptations was described in earlier studies in this series and in a recent review (13). Pyrex glass tubes, 35 mm. long and 5 mm. inside diameter, were used as reaction vessels. Constriction pipettes were used throughout. The optical measurements were made in a Beckman model DU spectrophotometer with Lowry-Bessey cuvettes, with or without Glick-Grunbaum inserts which use 6 μl. for a 1-cm. light path (14).

Micro Turbidimetric Procedure Adapted from Method of Tolksdorf et al.

Reagents—Bovine testicular hyaluronidase preparations were made by extraction at 3° of 0.5 gm. of fat-free dried testis powder with 100 ml. of 0.05 M phosphate buffer, pH 7, which was 0.05 M with respect to NaCl. The enzyme concentration was adjusted with the saline buffer to give a working solution which would produce a change in absorbance from between 0.6 and 0.7 to between 0.2 and 0.3 under the assay conditions which obtained. The Beckman instrument has maximal accuracy in this range (15). Human umbilical cord hyaluronate was also dissolved in the 0.05 M NaCl-phosphate buffer. The concentration was adjusted to give an initial assay turbidity equivalent to 0.6 to 0.7 absorbance. 0.05 M magnesium acetate was used so that the concentration in the final reaction mixture would be 0.01 M, the concentration found optimal to potentiate the nonspecific serum inhibitor under the conditions of the assay. 1 per cent bovine serum albumin (Fraction V, Armour and Company) in 0.5 M

* This investigation was supported by research grants, Nos. H2028 and RG 3911, from the National Institutes of Health, United States Public Health Service, and by a research grant from the Louis W. and Maud Hill Family Foundation.

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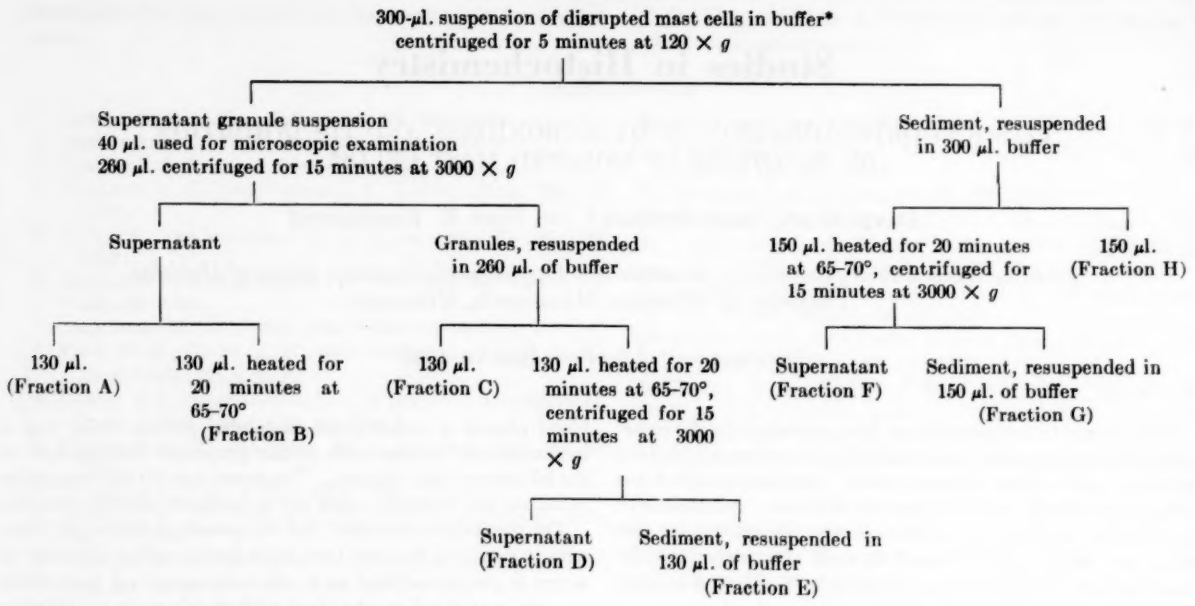
‡ Some of the data in this paper were included in a thesis submitted in partial fulfillment of the requirements of the Graduate School of the University of Minnesota for the degree of Master of Science.

DIAGRAM 1



* Potassium phosphate buffer, 0.05 M, pH 7.0, in all steps.

TABLE I

Hyaluronidase inhibition by mast cell fractions

Inhibitor preparation	Absorbance decrease	Inhibition	Inhibitor units per 10,000 cells
		%	
Control (without inhibitor)	0.343		
Fraction A	0.340	0	0
Fraction B	0.343	0	0
Fraction C	0.208	39	1.5
Fraction C (diluted 2 times)	0.295	14	
Fraction D	0.012	96	
Fraction D (diluted 2 times)	0.141	59	6.2
Fraction D (diluted 4 times)	0.221	38	
Fraction E	0.335	2	0.1
Fraction F	0.022	93	
Fraction F (diluted 2 times)	0.053	85	
Fraction F (diluted 4 times)	0.181	47	7.9
Fraction G	0.293	15	0.7
Fraction H	0.118	65	2.8
Fraction H (diluted 2 times)	0.246	29	

acetate buffer, pH 3.1, has been used, but more reproducible results were obtained with citrated blood plasma diluted 10 times with the acetate buffer, stabilized by heating according to Tolksdorf *et al.* (1), stored in the frozen state, and diluted 4 more times with the buffer before use. Heparin (100 units per mg.) was dissolved in 0.05 M phosphate buffer, pH 7.

Procedure—1 volume (1 to 10 μl.) each of magnesium acetate, enzyme, and inhibitor solution are mixed in reaction tubes in a 38° water bath and allowed to stand for 10 minutes (0.05 M phosphate buffer, pH 7, is substituted for inhibitor and magnesium solutions in normal enzyme assay). Add 1 volume of hyaluronate solution (prewarmed to bath temperature) to each tube, mix, and let stand for 15 minutes in the water bath. Remove the tubes from the bath, add 5 volumes of albumin or plasma protein solution, mix, and after 12 minutes measure absorbance at 400 mμ. For blank, substitute 0.05 M phosphate buffer, pH 7, for hyaluronate solution. For initial turbidity values add protein solution immediately after hyaluronate.

Remarks—Measurements were made at 400 mμ, rather than at 600 mμ, the standard in some laboratories, because the former gave higher absorbancies and also because color, which would be involved with the use of the longer wave length, was not present to interfere. Plots of absorbance against hyaluronate concentration in the assay mixture were linear up to an absorbance of 0.75 which was given by 0.5 mg. of the preparation used per ml. of enzyme reaction mixture. Inhibition by heparin was linear up to a concentration of 5 μg. per ml. of enzyme reaction mixture. This concentration gave 80 per cent inhibition. 1 unit of enzyme activity was defined as the amount of enzyme which would reduce the initial turbidity, 0.6 to 0.7 absorbance, by one-half in 30 minutes at 38°. 1 unit of inhibitor was the amount that inhibited 1 enzyme unit. In a series of 11 microdeterminations of the enzyme activity, the results lay within 4.5 per cent of the mean, and the standard deviation was 1.5 per cent.

Fractionation of Mast Cells

Mast cells were isolated from washings of peritoneal tissue from single rats according to the procedure described by Glick *et al.* (16). After centrifugation in the sucrose-Versene (the disodium salt of ethylenediaminetetraacetic acid)-phosphate density gradient, the cell layer was transferred to a small glass Potter-Elvehjem homogenizer tube and the cells were separated by centrifugation and washed with 1 ml. of Hanks' solution, again separated and washed with 1 ml. of 0.05 M phosphate buffer, pH 7, and finally resedimented and taken up in 200 μl. of the phosphate buffer. Although this buffer was slightly hypotonic, no effect on the cells was visible microscopically. The cell suspen-

sion was homogenized with an electrically driven pestle for 10 minutes, a period sufficient for disruption of practically all of the cells, and the pestle was rinsed with 100 μl. of the buffer. The combined homogenate and rinsing solution was treated as shown in the flow diagram, which depicts mast cell fractionation (Diagram 1). Since the sediment from each centrifugation was resuspended in the volume from which it was centrifuged, equal volumes of each fraction were comparable with one another. Negligible amounts were removed from the original cell suspension for cell counting in a hemacytometer.

Hyaluronidase Inhibition by Mast Cell Fractions

To obtain a solution which would inhibit within the range linear to inhibitor concentration under the conditions of the experiment, certain fractions were diluted with the buffer 2 and 4 times. (Linearity was maintained up to 80 per cent inhibition.) Decrease in absorbance without inhibitor was 0.34 to 0.43 in different experiments. Data of a typical experiment are given in Table I. The initial sediment which contained many granules had considerable inhibitor (Fraction H), but the heating solubilized the inhibitor and increased it about 3 times (Fraction F). The initial supernatant which contained suspended granules as the only bodies visible by phase contrast microscopy exhibited no soluble inhibitor (Fractions A and B), but the granules had inhibitor (Fraction C) which could be solubilized and increased approximately 4 times by the heating (Fraction D). Thus it seems that the granules possess inhibitor, which is not heat-labile. Moreover, heating sufficient to destroy the heat-labile labile inhibitor in serum actually liberates or otherwise increases the effect of the inhibitor from mast cells.

SUMMARY

The procedure of Tolksdorf *et al.* for the turbidimetric determination of hyaluronidase was adapted to a microscale requiring 0.01 to 0.001 the usual amount of sample.

The micromethod was applied to the measurement of hyaluronidase inhibition by fractions of mast cells isolated from peritoneal washings of the rat.

Inhibitor was found only in fractions containing granules. Inhibition by suspensions of granules was increased several-fold by heating at 65–70° for 20 minutes, conditions which destroy the nonspecific inhibitor of blood serum. Thus it is unlikely that the latter is derived from mast cells.

Acknowledgments—The authors wish to thank Gunhild Ottoson and Dr. Tatiana Ivanov for technical assistance.

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